CHARACTERISATION OF CELL POPULATION DATA AND ANGIOGENESIS IN MYELODYSPLASTIC SYNDROME

A DISSERTATION SUBMITTED IN PART FULFILLMENT OF THE REQUIREMENTS FOR THE M.D. DEGREE BRANCH III (PATHOLOGY) EXAMINATION OF THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY CHENNAI TO BE HELD IN APRIL 2015.

CERTIFICATE

This is to certify that this dissertation titled **"CHARACTERISATION OF CELL POPULATION DATA AND ANGIOGENESIS IN MYELODYSPLASTIC SYNDROME"** is a bonafide work done by Dr. Vishnu Chandra Kumar. A, in part fulfilment of the rules and regulations for the M.D. Branch III (Pathology) Degree Examination of the Tamil Nadu Dr. M.G.R Medical University, to be held in April 2015.

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ACKNOWLEDGEMENT

I express my warm thanks to Dr. Joy Mammen (Transfusion Medicine and Immunohaematology), Dr. Marie Therese Manipadam (General Pathology), Dr. Biju George (Clinical Haematology) and Dr. Sukesh Chandran Nair (Transfusion Medicine and Immunohaematology) for their guidance and persistent help for the completion of this dissertation.

ABBREVIATIONS:

MDS	-	Myelodysplastic syndrome.
CPD	-	Cell population data.
MVD	-	Microvessel density
RCUD	-	Refractory Cytopenia with Unilineage Dysplasia
RCMD	-	Refractory Cytopenia with Multilineage Dysplasia
RARS	-	Refractory anaemia with ring sideroblasts.
VCS	-	Volume Conductivity Scatter
VEGF	-	Vascular Endothelial Growth Factor (VEGF)
bFGF	-	basic Fibroblast Growth Factor
MA	-	Megaloblastic anaemia.
UMALS	-	Upper median angle light scatter.
LMALS	-	Lower median angle light scatter.
LALS	-	Lateral angle light scatter.
AL	-	Axial light scatter.
MNV	-	Mean neutrophil volume.
MNC	-	Mean neutrophil conductivity.
MNS	-	Mean neutrophil scatter.
MLV	-	Mean lymphocyte volume.
MLC	-	Mean lymphocyte conductivity.
MLS	-	Mean lymphocyte scatter.
MMV	-	Mean monocyte volume.
MMC	-	Mean monocyte conductivity.
MMS	-	Mean monocyte scatter.
MEV	-	Mean eosinophil volume.
MEC	-	Mean eosinophil conductivity.
MES	-	Mean eosinophil scatter.

PPHA	-	Pseudo Pelger Huet Anomaly.
IPSS	-	International Prognostic Scoring System.
FAB	-	French-American-British
AA	-	Aplastic Anaemia
RA	-	Refractory Anaemia.
RN	-	Refractory Neutropenia.
RT	-	Refractory Thrombocytopenia.
AML	-	Acute Myeloid Leukaemia
ALL	-	Acute Lymphoid Leukaemia
HIF 1a	-	Hypoxia Induced Factor 1a

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ABSTRACT

TITLE OF THE ABSTRACT **:** CHARACTERISATION OF CELL POPULATION DATA AND ANGIOGENESIS IN MYELODYSPLASTIC SYNDROME.

DEPARTMENT **:** PATHOLOGY

NAME OF THE CANDIDATE **:** VISHNU CHANDRA KUMAR. A

DEGREE AND SUBJECT **:** MD. PATHOLOGY

NAME OF THE GUIDE **:** DR. JOY MAMMEN

OBJECTIVES:

1. To look for differences in the cell population data (CPD) values and differences in the micro vessel density (MVD) in the bone marrow in cases of Myelodysplastic syndrome (MDS).

METHODS:

The Cell Population Data (CPD) values obtained from the automated cell counter (Beckman Coulter DxH800) of 68 cases with MDS were compared with 155 non MDS patients and 98 normal healthy blood donors. The microvessel density in trephine biopsies of 101 MDS cases was counted using immunohistochemistry (antiCD34 antibody) by “hotspot” method and was compared with 35 normal controls.

RESULTS:

There was a significant increase in the mean neutrophil volume and mean monocyte volume in MDS cases compared to non MDS and healthy donors (p-value: 0.000 and 0.000 respectively). A significant decrease in the neutrophil scatter and eosinophil scatter was noted

in cases of MDS in comparison to non MDS and normal controls (p-value=0.000 and 0.000) respectively). A significant increase in the bone marrow mean MVD in cases of MDS was noted compared to the control marrows (p-value=0.000).

CONCLUSION: There is a measurable difference in the cell population data (CPD) in cases of MDS which can be detected at an early stage even before a bone marrow examination is carried out. The increased MVD in MDS is a potential target for antiangiogenic therapy.

KEYWORDS: Myelodysplastic syndrome (MDS), Cell population data (CPD), Automated Beckman Coulter DxH800 haematology analyser, Microvessel density (MVD).

INTRODUCTION:

Myelodysplastic syndromes are a heterogenous group of hematopoietic disorders characterised by ineffective erythropoiesis in the bone marrow leading to decreased peripheral blood counts and their clinical consequences. They are premalignant disordersand can progress to acute leukaemia. These disorders are chronic in nature, resistant to treatment and cause considerable morbidity and mortality. The precise nature and pathophysiology of this group of disorders is currently under active research.

The current diagnostic modalities for MDS include examination of peripheral blood film, bone marrow study and cytogenetic analysis. The morphological changes of the blood cells, especially in white blood cells can be picked up in the automated haematology analysers in addition to the labour intensive manual blood smear examination. The modern cell counters such as the Beckman Coulter DxH800 use the VCS technology (mean cell channel for Volume, Conductivity and Scatter) to provide a five part differential leucocyte count. The data acquired by the automated cell counter through the VCS technology is collectively known as the Research population data or the Cell Population Data. (CPD data). The present study aims to look fordifferences in the CPD in cases with MDS since this can spark an early clue to the presence of MDS even before a manual smear examination is carried out.

The subsequent part of the study aims at assessing the degree of angiogenesis in the marrow of patients with MDS. Being a preleukemic condition, development of an increased degree of angiogenesis has been linked to a dangerous leap towards transformation to malignancy. A precise understanding of the degree and patterns of angiogenesis can guide patient care and management with antiangiogenic therapies.

REVIEW OF LITERATURE:

DEFINITION:

Myelodysplastic syndromes (MDS) are clonal disorders of hematopoietic stem cells. They are characterized by ineffective haematopoiesis resulting in marrow failure, peripheral blood cytopenia(s) and dysplasia in one or more of the major myeloid cell lines. It is a preleukemic condition with a propensity to progress into acute myeloid leukaemia (AML). (1)

HISTORY:

MDS are a heterogeneous group of haematological disorders which have significant morbidity and mortality. There are quantitative and qualitative alterations within one or more of the hematopoietic cell lines leading to progressive bone marrow failure**.**In 1923, an Italian physician named Giovanni Di Guglielmo described a set of bone marrow diseases which were associated with anaemia and bizarrely shaped red blood cells. This was called Di Guglielmo syndrome. It was noted that unlike pernicious anaemia the anaemia in this condition was resistant to liver therapy. Some cases that were once called Di Guglielmo syndrome could now be called MDS.In the early 20th century, it was recognised that a considerable number of persons with acute myeloid leukaemia had a preceding period of anaemia and abnormalities in the cell production. These conditions were together recognised under the term "refractory anaemia". This term was used because attempts to treat the anaemia by replacement of the necessary nutrients did not succeed in cure and the patients remained unresponsive to all the active hematinics then known. The term "preleukemia" was used by Block et al to include a set of patients with ill-defined and varied haematological conditions that terminated in the development of acute leukaemia. (2)

In the late 1950s, William Dameshek proposed that even a small increase in the number of blasts in the bone marrow indicated the development of acute myeloid leukaemia(s). He coined the term smoldering leukaemia or low percentage leukaemia to identify this set of patients. He linked various forms of refractory anaemia to the development of acute leukaemia(s).(3)

In the year 1976, theFAB classification published and popularised the term myelodysplastic syndrome (MDS).

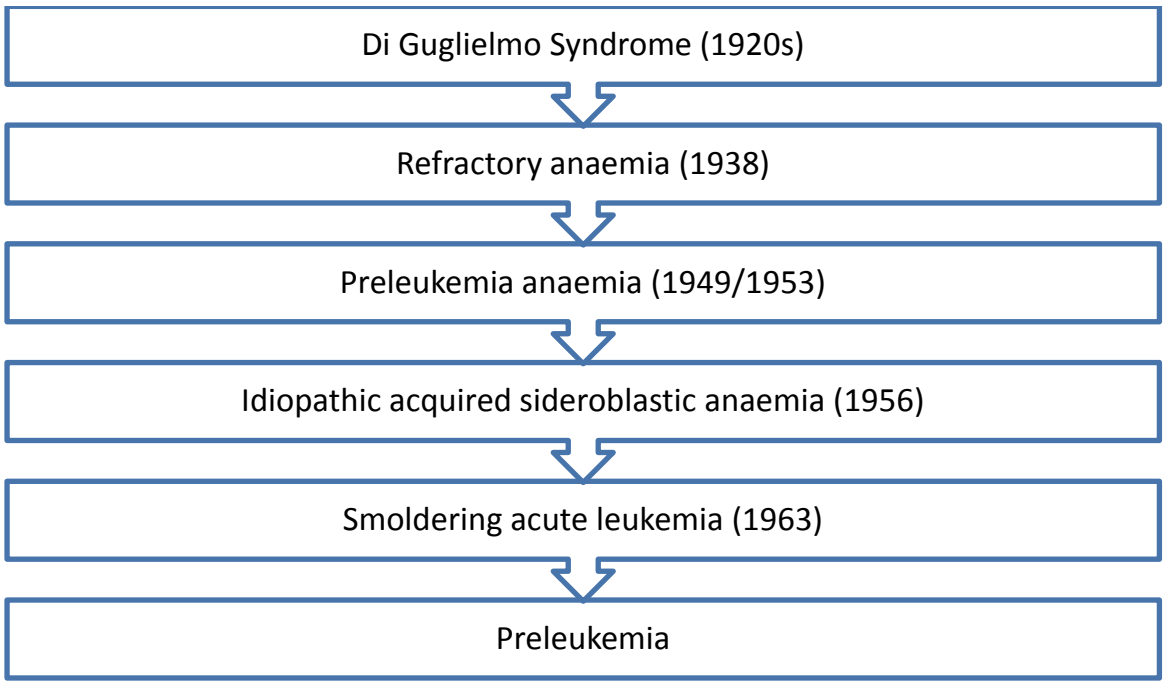

Figure 1: Early history of Myelodysplastic Syndromes (MDS)

ETIOLOGY:

MDS can occur either as primary (de novo) disorders or secondary to previous treatment with chemotherapy, radiation therapy or immunosuppressive drugs in persons with organ transplantation or autoimmune disorders. Environmental and occupational factors like ionising radiation, smoking, pesticides and industrial solvents such as benzene are associated with an increased risk for primary myelodysplastic syndromes. Family history of haematological neoplasms and inherited hematologic disorders like dyskeratosis congenita, Fanconi anaemia, Diamond-Blackfan syndrome and Shwachmann-Diamond syndrome are also associated with an increased risk for primary MDS. (1)

Therapy related MDS accounts for about 10% of all cases of MDS and has a poor prognosis compared to primary MDS.(4) The common chemotherapeutic drugs that cause therapy related MDS are alkylating agents and topoisomerase II inhibitors.

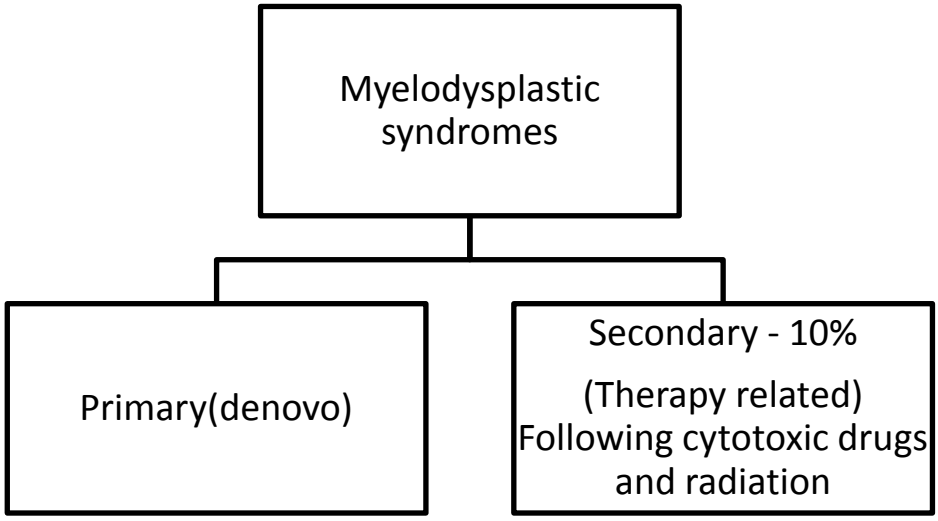

Figure 2: Etiological classification of MDS

EPIDEMIOLOGY:

MDS is a disease of the elderly. Epidemiological data are available at present from large scale studies mainly from the western countries. Studies in the western population indicate that the peak incidence of myelodysplastic syndromes is seen in the sixth to seventh decade of life. (1)(5) Studies published regarding the prevalence and epidemiology of MDS in India have pointed to differences in the epidemiology of MDS between Indians and the western population. The peak incidence of MDS appears to be a decade earlier in the Asian population, the exact reasons for which are not known. Factors such as environmental pollution, exposure to pesticides and toxic chemicals are believed to play a causative role. (6)(7)

The non-age corrected annual incidence of MDS is 3-5/ 100000. But, the incidence rises to >20/100000 amongst those older than 70 years. Men are affected more than women. (1)

CLINICAL FEATURES:

MDS display clinical heterogeneity. Patients with MDS can be asymptomatic. The peripheral blood cytopenia(s) may be an incidental finding noted in a routine blood count. The clinical features range from an indolent disease with near-normal life expectancy to an aggressive behaviour overlapping with acute myeloid leukaemia. MDS isgenerally refractory to treatment and the majority of patients develop complications such as bone marrow dysfunction or hematopoietic neoplasm.(5)

Anaemia is the most common cytopenia associated with MDS. It is found in approximately 80% - 85% of patients.(8) Patients with anaemia manifest pallor, shortness of breath, fatigue, chest pain and palpitations exacerbated by exertion. Thrombocytopenia is seen in 35% - 45%

and manifests as easy bruising, mucocutaneous bleeding, purpura or petechiae. Gastrointestinal bleeding and intracranial haemorrhage may occur in severe cases.(8) Neutropenia manifests as recurrent bacterial infections. Organomegaly is uncommon in MDS. It can be present in cases with overlapping features of MDS and chronic myeloproliferative disorders.

Clinical Scoring Systems:

Scoring systems are used for MDS rather than staging systems. These scoring systems are based upon:

- The number of myeloblasts in the bone marrow.
- The number and degree of cytopenia(s).
- Karyotypic abnormalities.

Several investigators have attempted scoring systems using different parameters such as platelet count, haemoglobin, blast count and cytogenetic features.(9)(10) The most commonly used scoring systems are the International Prognostic Scoring System (IPSS) and the WHO Classification-based Prognostic Scoring System (WPSS). The latter system also takes into consideration the transfusion burden.(11)

The International Prognostic Scoring System (IPSS) was proposed by Greenberg and associates(12)in 1997. The IPSS includes blast percentage, karyotype of the bone marrow and the number of cytopenia(s) in the peripheral blood. The recommended thresholds for cytopenia(s) are haemoglobin <10g/dl, absolute neutrophil count <1.8x10^9/L and platelet count<100x10^9/L. However, if the values are above these levels, MDS can still be considered if definite morphological or cytogenetic evidence is present.(1)

Few investigators also use the term “low grade” and “high grade” MDS. This division is based upon a bone marrow blast count of less than 5% and 5 or more than 5% respectively. This is not a routinely used grading scheme for MDS.(11)

CLASSIFICATION OF MYELODYSPLASTIC SYNDROME:

A succession of classification systems for MDS has been developed. The aim of these classification systems is to predict the overall survival and risk of progression to acute myeloid leukaemia.The classification of MDS was initially based on the French-American-British (FAB) classification. The first FAB classification of MDS was proposed in the year 1976 under the term “dysmyelopoietic syndromes”. This initial classification had only two categories namely, RAEB and Chronic myelomonocytic leukaemia (CMML). This was further revised in the year 1982.The revision included 5 categories which were RA, RARS, RAEB, RAEB-t and CMML (Table 2). The FAB classification was based entirely on findings recorded by cytological analysis of peripheral blood smear and bone marrow aspirate. The major criteria that were used for this classification of MDS were the percentage of blasts in the peripheral blood and bone marrow aspirates and the presence of dysplastic changes in the different bone marrow cell lines. (13)

A more comprehensive approach for the classification of MDS was proposed by the World Health Organization (WHO) in the year 1999 to 2001, by incorporating changes in the 1982 FAB classification. This early WHO (2001)classification system described MDS under 10 categories. The alteration that was made include the following: RCMD, RCMD-RS, MDS-U, MDS with 5q (Del) and therapy related MDS were newly added. RAEB of FAB classification was split into RAEB-1 and RAEB-2. The category of RAEB-t was removed. CMML was moved to MDS/MPD category.

The WHO 2001 classification was further revised in the year 2008. This classification is currently in use. Presently there are 11 categories of MDS under this recent classification. Refractory neutropenia (RN), Refractory thrombocytopenia (RT) were considered along with refractory anaemia (RA) to comprise the category of RCUD. A new category of childhood MDS was added. RCMD and RCMD-RS were merged as one group.

The WHO 2008 classification highlights the importance of integrating histologic examination of the bone marrow aspirate and the trephine biopsy with other diagnostic techniques such as cytogenetic analysis and molecular genetics, in the context of relevant clinical information.

The WHO classification system recommends that at least 10% of the cells in a particular cell lineage should be morphologically dysplastic in the bone marrow to declare a dysplasia of that particular lineage.(1)(13)In particular, the extent of dysplasia, whether it is unilineage or multilineage, has an important role in the WHO classification. This feature was not considered in the FAB system.(13)

Recently, transfusion dependence has been identified to have an effect on survival of patients with MDS. As mentioned earlier, the consideration of transfusion dependency within the WHO system has produced the WHO classification–based prognostic scoring system.(13)

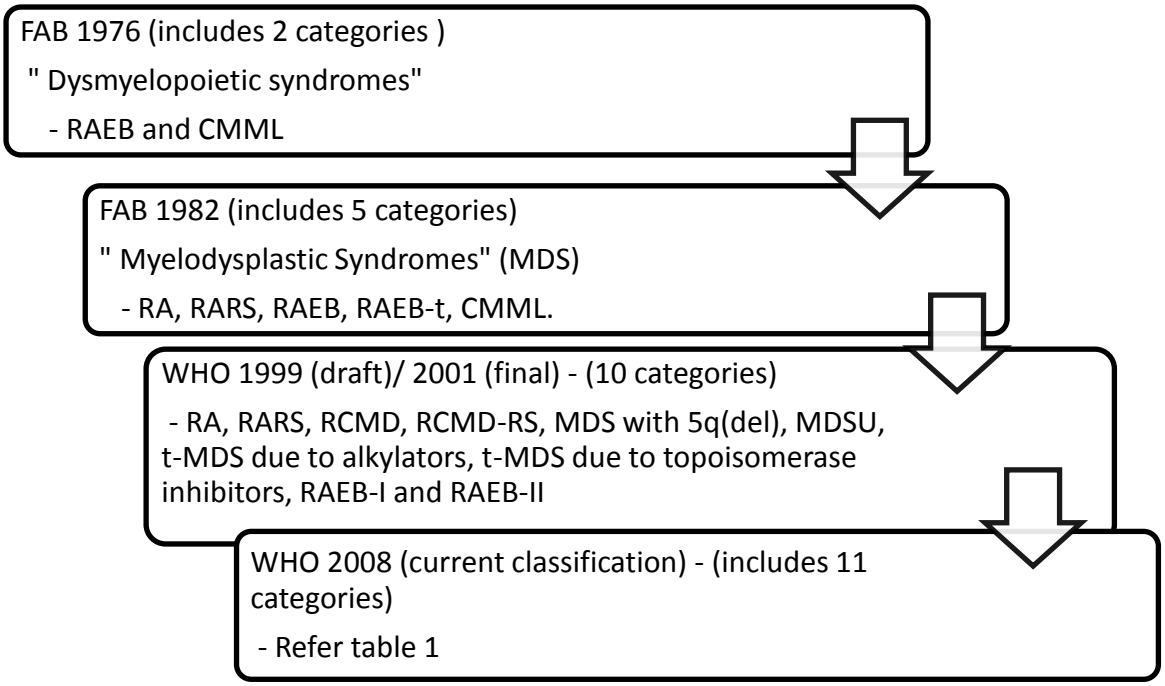

Figure 3: Evolution of classification of MDS

Table 1: Myelodysplastic syndrome entities according to the WHO 2008 classification.

Refractory cytopenia with unilineage dysplasia (RCUD)

- Refractory neutropenia.
- Refractory anaemia.
- Refractory thrombocytopenia.

Refractory anaemia with ring sideroblasts (RARS)

Refractory cytopenia with multilineage dysplasia (RCMD)

Refractory anaemia with excess blasts(RAEB)

- RAEB-1
- RAEB-2

MDS with isolated del (5q) abnormality.

MDS, unclassified.

Therapy-related MDS (t-MDS)

Table 2: Differences between the FAB 1982 classification and the WHO 2008 classification.

FAB CLASSIFICATION(1982) **(Old system)**	**WHO SYSTEM(2008)** **(New system)**
Refractory anaemia (RA)	Refractory cytopenia with unilineage dysplasia (RCUD) • Refractory neutropenia. • Refractory anaemia. • Refractory thrombocytopenia.
Refractory anaemia with ringed sideroblasts (RARS)	• Refractory anaemia with ring sideroblasts (RARS)
	Refractory cytopenia with multilineage dysplasia (RCMD) including RCMD-RS.
Refractory anaemia with excess blasts(RAEB)	Refractory anaemia with excess blasts I (RAEB I) : 5-9% blasts. Refractory anaemia with excess blasts II (RAEB II): 10-19% blasts
Refractory anaemia with excess blasts in transformation (RAEB-T)	RAEB-T has been eliminated. It is now considered as acute leukaemia.
Chronic myelomonocytic leukaemia (CMML)	Removed from MDS. It is considered under the new category myelodysplastic-myeloproliferative overlap syndromes.
	5q- syndrome
	MDS-U (myelodysplastic syndrome unclassifiable)
	Refractory cytopenia of childhood (RCC)

APPROACH TO THE DIAGNOSIS OF MDS:

The routine diagnostic workup of MDS includes a thorough clinical examination followed by the morphologic evaluation of peripheral blood smear, marrow aspirate and bone marrow trephine biopsy, which are interpreted in correlation with marrow cytogenetic results.(13)

The correlation with marrow cytogenetics is also essential. The karyotype of the marrow is essential for the risk stratification and for the calculation of the IPSS score. However only 50% cases of MDS show cytogenetic abnormalities. Hence, the presence of a normal karyotype does not exclude a diagnosis of MDS. On the contrary, an abnormal karyotype may indicate MDS within an appropriate clinical context. (13) The role of cytogenetics in the diagnosis of MDS is discussed in the following sections. (page no: 29 – 31)

PERIPHERAL SMEAR EXAMINATION:

The peripheral blood smear in cases of MDS almost always shows some evidence of cytopenia. The red blood cells are macrocytic and the red cell distribution width(RDW) is often increased.(11) Dimorphic red blood cells with a combination of both macrocytes and hypochromic microcytes may be seen when there are significant ring sideroblasts in the marrow.(11) A detailed cytomorphological analysis of 3156 MDS patients by Ulrich germing *et al* showed that, the most frequent features of dyserythropoiesis in the peripheral smear were anisocytosis, poikilocytosis and polychromasia. Patients with RARS subtype have more pronounced features of dyserythropoiesis in the peripheral blood.(14)

Dysplastic features in the granulocytes are more easily appreciated in a peripheral smear than the marrow aspirate. The most frequent dysplastic sign in granulocytic lineage was found to

be pseudo-Pelger cells followed by left shift in the white cell precursors and hypogranularity.(14)

Anisometry of the platelets is also a frequent finding in all the subtypes of MDS.(14) Approximately 66 percent of patients have signs of dysplasia in the peripheral smear and 34 percent patients do not show signs of dysplasia in a peripheral smear examination.(14)

A clear relation between the presence of any particular dysplastic sign and the peripheral cell count has not been reliably demonstrated. However, low cell counts can be associated with increasing number of signs of dysplasia in the marrow.(14)

BONE MARROW ASPIRATE:

This is a critical tool for diagnosis and subtyping of MDS. The percentage of blasts and degree of unilineage or multilineage dysplasia should be recorded. Dysplasia should be present in at least 10% of the cells of any lineage.(1)(11)

The cytopenia(s) in the peripheral blood are thought to occur due to an enhanced degree of apoptosis in the bone marrow precursor cells. (1)The majority of patients have a norm cellular or a hypercellular marrow. 4-16% of patients also present with a hypocellular marrow many of whom are in the RCUD category.(14)

The features of dyserythropoiesis include nuclear budding, nuclear hyperlobation, multinuclearity, internuclear bridging, karyorrhexis, cytoplasmic vacuoles and cytoplasmic basophilic stippling. The presence of ring sideroblasts is another manifestation of dyserythropoiesis and is defined as at least five siderotic granules, covering at least one third of the circumference of the nucleus.(1) Ring sideroblasts are not specific for MDS. It can also

be seen in non-neoplastic conditions such as alcoholism, sideroblastic anaemia and due to certain drugs.

Megaloblastoid change/ nuclear-cytoplasmic asynchrony is also considered as a feature of dyserythropoiesis. But this alteration can also be seen in non-MDS samples and hence needs to be interpreted with caution and should be correlated with other dysplastic features.

Marked erythroid hyperplasia(50% or more) can be seen in about 15% of patients with MDS. These myelodysplastic syndromes with erythroid predominance comprise a small, but a significant number of myelodysplastic syndromes.(15) Erythroid hyperplasia is more frequent in RARS and RCMD with ring sideroblasts. (14) On the contrary, 5% of MDS cases can also show marked erythroid hypoplasia or aplasia.(11)In the erythroid precursors, the nuclear changes of dysplasia are more frequent than the cytoplasmic changes.(14)

The features of dysgranulopoiesis include nuclear hypolobation such as pseudo-Pelger-Huet anomaly (PPHA), hypersegmentation of the nuclei at inappropriate stage of maturation and alterations in the granularity of the cytoplasm (Hypergranularity, hypogranularity or agranularity). Identification of dysplasia in the myeloid cells can be challenging in cases of MDS when there is a left shifted myeloid maturation.(1)(11)

Pseudo Pelger Huet anomaly is characterised by round or oval, peanut shaped nuclei or bilobed nuclei with abnormally clumped chromatin in the neutrophils. It resembles the true Pelger Huet anomaly which is an autosomal dominant disorder with normal neutrophil function. PPHA anomaly is an acquired abnormality and is commonly seen in MDS and myeloid neoplasms. Traditionally, PPHA is associated with myelodysplasia. It represents

severe degree of dysplasia in MDS cases and is associated with other abnormalities seen in MDS. (16)

PPHA can also occur due to infections or due to medications such asMycophenolate mofetil, Tacrolimus, gancyclovir, fluconazole etc. used in conditions such as bone marrow transplantation or solid organ transplantations. .PPHA occurring in non-neoplastic conditions may be transient and is reversible. Factors such as spontaneous resolution of the anomaly, normalisation in the segmentation of the neutrophils and alterations in the proportion of neutrophils according to the dose adjustment of medication are helpful in attributing a non neoplastic cause to the PPHA. (16)

An isolated PPHA without underlying MDS or other myeloid neoplasms usually have a higher proportions of PPHA cells (>30%) and are transient. In MDS, there are lesser number of PPHA cells in the peripheral circulation, but these are persistent and are not easily reversible.(16)

Eosinophilia in the bone marrow has been identified in about 10% of the cases of MDS. MDS with eosinophilia is a rare entity and is yet not classified as a separate category in the current WHO 2008 classification. A study on 145 patients with denovo myelodysplastic syndromes by Matsushima *et al* documented certain features observed in the eosinophils in MDS which were ring shaped nucleus, disproportion in eosinophilic granule content, basophilic granules and vacuolation in the cytoplasm. In addition to these morphological abnormalities, 62% of patients who had eosinophilia in the setting of MDS have major karyotypic abnormalities.(17)

Studies have shown a poor survival in MDS patients who have an increased proportion of bone marrow eosinophils and basophils. (18) On the contrary, few other case reports have

also recorded an indolent behaviour of MDS in the presence of persistent eosinophilia. (19) Hence the exact significance of eosinophilia and its clinical impact is unclear.

The features of dysmegakaryopoiesis include micromegkaryocytes with hypolobated nuclei, pleomorphism in the size with monolobated nuclei. "Pawn ball megakaryocyte" is a form of dysmegakaryopoiesis that is used when the megakaryocyte has multiple widely separated nuclei. Dysplasia in the megakaryocytic lineage is better appreciated in the trephine biopsy sections. It helps to verify the findings seen on the BM aspirate smears.(11) An assessment should be made on atleast 20 to 30 megakaryocytes when possible and in both the biopsy sections and aspirate smears. Dysmegakaryopoiesis is extremely rare in the unilineage dysplasia types. The features of dysplasia in megakaryocytic lineage is more frequent in the more advanced types.(14)

The study done by Germing U *et al*showed that pseudo Pelger cells, hypo granulation, mononuclear megakaryocytes as well as micromegakaryocytes were the most important signs of non erythroid dysplasia and 85% percent of cases of RCMD could be diagnosed by the identification of these four parameters of dysplasia.(14) The presence of ring sideroblasts, platelet anisometry and a left shifted granulopoiesis are few uniform features of dysplasia that is seen in almost all cases of MDS.(14)

MDS cases with unilineage dysplasia and absence of ring sideroblasts is difficult to diagnose on the basis of cytological assessment alone in the absence of clonal cytogenetic markers. In these instances, the limitations of cytology is obvious.

BONE MARROW TREPHINE BIOPSY:

In MDS, the bone marrow is usually hypercellular. The hypercellularity is thought to result from an increased production of the marrow cells to compensate for the peripheral

cytopenia(s). The topography of the marrow is considerably distorted. The stromal alterations include an altered cellular distribution of the constituent hematopoietic tissue with notable differences in the size of the hematopoietic islands, increase in the number of histiocytes, increasedmarrow vasculature, poorly formed erythroid islands, pleomorphism in adipocyte size and increase in the reticulin content.

Though hypercellularity is the usual picture, a significant proportion (approximately 10%) of patients with MDS also have hypocellularity of the marrow. These cases of hypoplastic MDS can be potentially confused with a hypoplastic marrow. (1)

In many instances, it may be very challenging to distinguish hypocellular MDS from aplastic anaemia (AA). Macrocytosis of the red blood cells may be found in both the conditions. The presence of neutrophils with a pseudo-Pelger Huet anomaly with or without a hypogranular cytoplasm favours a diagnosis of MDS over AA.(11)In a case of hypoplastic MDS, the bone marrow biopsies show scattered dysgranulopoiesis, lesser degrees of dysmegakaryopoiesis and patchy islands of dyserythropoiesis. There may be an uneven distribution in the cellularity wherein discernible dysplastic islands of hematopoietic cells are seen admixed with completely acellular areas.

CD34+ve cells are decreased in marrows with AA. Though clonal cytogenetic abnormalities are identified in cases of MDS at the time of presentation, they can also be identified occasionally in cases of AA. 10-15% of cases with AA, who are not treated with hematopoietic stem cell transplantation develop MDS probably because of an independent clonal proliferation of cells with an acquired selective growth advantage(11)

The expression of CD34, a marker of early progenitor cells, is increased in cases of MDS irrespective of the MDS subtype. Thus, immunohistochemistry can aid in the diagnosis of MDS, especially in cases with fibrotic or hypocellular bone marrow. It also assists in the

identification of Abnormal Localisation of Immature Precursors(ALIPs) which when present is correlated with an increased risk of transformation of MDS to acute leukaemia. (11)

Dysplastic megakaryocytes in certain cases of MDS also show positive staining for CD34. In a study performed by Tang et al, they observed that in about 14% cases of MDS, there was expression of CD34 by megakaryocytes. The expression of CD34 by the megakaryocytes in cases of MDS correlated with severe cytopenia, increased number of myeloblasts, presence of high risk cytogenetic abnormalities and reduced overall survival. This study concluded that expression of CD34 by megakaryocytes was a strong and an independent prognostic factor in cases of MDS.(20)

Generally, the reason for the presence of cytopenia in the peripheral blood is believed to be due to increased apoptosis of the progenitor cells in the bone marrow. Electron microscopic studies in a case with trilineage dysplasia showed majority of the hematopoietic cells with ultrastructural features of apoptosis such as margination and condensation of nuclear chromatin, nuclear pyknosis, vacuolisation of cytoplasm and polarisation of the organelles. Hence the hypercellularity of the bone marrow appears to be a direct consequenceof an increased rate of apoptosis.

Matsuda *et al* demonstrated that there was no correlation between the dysplastic lineage and the type of peripheral cytopenia in patients with unilineage dysplasias. (21)To elaborate further, there was no correlation between the peripheral blood absolute neutrophil counts and dysgranulopoeisis in the marrow. Hence, dysgranulopoeisis alone could not be considered as the only reason for a low absolute neutrophil count. So, dysplastic features in the granulocytic cells might be unrelated to ineffective erythropoiesis caused by increase in the rate and amount of apoptosis. (21)

Interestingly, in patients with dysmegakaryopoiesis, the peripheral platalet counts were higher than in those cases that did not have dysmegakaryopoiesis of 10% or more. This is similar to the pathology of 5q deletion syndrome, where the peripheral platelet counts are either normal or increased, inspite of the bone marrow harbouring megakaryocytes with >10% dysplasia.

Moreover, 55% of patients with dysmegakaryopoiesis of 40% or more, had increased number of megakaryocytes. A similar correlation was present between the number of megakaryocytes and the presence of micromegakaryocytes. 75% of patients who had micromegakaryocytes in the marrow had an increased number of megakaryocytes. This study gives an indication that dysplasia may not completely correlate with an increased amount of apoptosis as believed widely.(21)

IMPORTANCE OF ENUMERATION OF BLASTS IN MDS:

An accurate assessment of the percentage of blasts in the bone marrow and peripheral smear is critical for the diagnosis, subtyping, risk stratification and assessment of treatment response in MDS. In certain studies, the percentage of blasts have been taken as one of the most important prognostic indicator.(13)

Immature cells to be included while counting blasts include myeloblasts, monoblasts, and megakaryoblasts. The blast count derived from the aspirate should be correlated with the estimate from the biopsy sections.(13)For accurate blast enumeration, a 500 cell differential count is required.

A change in the number of blasts can change the disease classification and predict clinical course and outcomes.(11) Circulating blasts and immature cells are also seen in those patients receiving growth factor therapy, in actively regenerating marrow, sepsis causing acute stress in the marrow and in those patients who have undergone a recent stem cell transplantation.(11)

In trephine biopsies, blasts may be identified using immunohistochemical stain for CD34. (13) However, it must be remembered that not all blasts are positive for CD34 and hence it should not be used as a substitute for careful morphological assessment. Markers that can be used to visualise CD34 negative blasts are CD117, lysozyme and CD68. Myeloperoxidase (MPO) is often weak or negative in the blasts seen in MDSs. Flow cytometry may help in assessing the frequency of marrow blasts and to confirm their immunophenotype. (13)

The natural course of the disease, in many cases of MDS is progression to acute leukaemia. The proportion of cases with such transformation varies depending on the subtype of MDS. Persons with complex cytogenetic abnormalities or with subtypes such as RAEB-2 have a 50% life time risk of transformation to acute leukaemia while “low grade” subtypes such as refractory cytopenia with unilineage dysplasia carry a life time risk of <5%.(22)

Acute leukaemia arising from an underlying MDS is almost always of the myeloid lineage. Patients of MDS progressing to acute leukaemia develop complex cytogenetic abnormalities at the time of transformation. Those cases that have an initial response to treatment might acquire a second cytogenetic abnormality, become refractory to treatment and evolve into acute leukaemia. However, rare cases of MDS with transformation to ALL have also been documented in case reports.(23)(24)

According to the WHO system of classification (2008), the subtyping of MDSis not based only on morphologic findings. It also includes the use of clinical, genetic, immunophenotypic, and biologic information to define separate disease entities.Since, a single biologic or genetic marker that identifies all or most cases of MDS has not been discovered till date, bone marrow morphologic assessment is important for subtyping the majority of cases of MDS. (13)

REFRACTORY CYTOPENIA WITH UNILINEAGE DYSPLASIA:

This category encompasses those MDS cases which present with a refractory cytopenia restricted to a single hematopoietic cell lineage and includes refractory anaemia (RA), refractory neutropenia (RN) and refractory thrombocytopenia (RT). (1)

RCUD comprises of 10-20% of all the cases of MDS. Vast majority of RCUD cases are - refractory anaemia. Refractory neutropenia and refractory thrombocytopenia are rare. (1)This designation encompasses the MDS cases that manifest with refractory cytopenia and associated with dysplasia limited to 1 cell line.

Refractory bicytopenia can also be included in this category if accompanied by unilineage dysplasia. The type of dysplasia in most of the cases corresponds to the type of cytopenia, e.g. anaemia and erythroid dysplasia. The presence of blasts in the peripheral blood, generally excludes a diagnosis of RCUD..Refractory pancytopenia with unilineage dysplasia is best considered under MDS-U (MDS unclassifiable) because of the uncertain biology of the findings.

Usually, the marrow is hypercellular and shows erythroid hyperplasia. Dyserythropoiesis is present, but ring sideroblasts are fewer than 15% of the erythroid cells. In the absence of

cytogenetic abnormality, it requires cytopenia(s) for at least 6 months and the exclusion of reactive causes to make a diagnosis of RCUD.(13)

Persons with RCUD have an indolent clinical course with a median survival of approximately 66 months and a 2% rate of progression to AML at 5 years follow up.(1) It is generally considered a "low grade" MDS. In cases characterized by anaemia and dyserythropoiesis, conditions such as megaloblastic anaemia and congenital dyserythropoietic anaemia must be carefully excluded.

REFRACTORY ANAEMIA WITH RING SIDEROBLASTS: (RARS)

RARS is characterized by unexplained anaemia, and dysplasia involving the erythroid lineage with ring sideroblasts constituting >15% or more of the erythroid precursors. The dysplasia is confined to the erythroid cells. Anaemia is the predominant finding. The criteria are very similar to those described for RA, except that there are 15% or more of ring sideroblasts in the bone marrow. Ring sideroblasts can be seen in any of the subsets of MDS. AML and certain non-neoplastic conditions also show ring sideroblasts in the marrow. Hence, secondary causes of ring sideroblasts should be excluded before the diagnosis of RARS is made. Importantly, the myeloblasts should comprise <5% of the nucleated cells in the bone marrow and are usually not seen in the peripheral blood.(1)

RARS is also considered a "low-grade" MDS. It is reported to have the best prognosis and lowest rate of conversion to AML among all the subtypes of MDS with a median survival of 7 to 9 years or longer. Approximately 1-2% of cases of RARS transforminto AML.(1)Ring sideroblasts are also seen in other subtypes of MDS such as RAEB and RCMD. Non neoplastic causes of ring sideroblasts include alcohol, toxins such a lead and benzene, drugs such as isoniazid, zinc supplementation and copper deficiency.(1)In rare occasions, the

platelet count is increased (≥450 × 103/μL), and the differential diagnosis between a myeloproliferative disorder becomes problematic. (13)Most of these cases may be assigned to the provisional entity of RA with ring sideroblasts and thrombocytosis, which is currently considered within the MDS/MPN group.(13)

REFRACTORY CYTOPENIA WITH MULTILINEAGE DYSPLASIA:

This category was not recognized in the FAB system and has been added in the WHO classification. This subtype accounts for ~30% cases of MDS. RCMD is characterized by one or more cytopenia(s) in the peripheral blood and dysplasia involving 2 or more of the myeloid lineages: erythroid, granulocytic, and/or megakaryocytic lineages. There are less than 1% blasts in the blood and less than 5% in the bone marrow. Auer rods are not seen. If 15% or more of the erythroid precursors are ring sideroblasts then the diagnosis of RCMD with ring sideroblasts should be made. (13)

Cases that satisfy the criteria for RCMD but consistently have 1% blasts in the blood should be classified MDS unclassifiable, and those cases with multilineage dysplasia and lesser than 5% blasts in the bone marrow but 2% to 4% blasts in the blood should be classified as RAEB-1. (13)The frequency of evolution into acute leukaemia at two years is ~10% for cases with RCMD.

REFRACTORY ANEMIA WITH EXCESS BLASTS:

RAEB is a myelodysplastic syndrome with 5% - 19% blasts in bone marrow or peripheral blood. However, if there are lesser than 5% blasts in the bone marrow, the presence of 2% - 4% blasts in the peripheral blood is adequate for the diagnosis of RAEB-1. There are two

subcategories that are recognized based on the differences in survival and incidence of evolution into acute leukaemia:

RAEB-1, defined as having 5% to 9% blasts in the bone marrow or 2% to 4% in the blood.

RAEB-2, more than 10% blasts in the marrow and/or 5% or more in the peripheral blood. The presence of Auer rods shifts the classification to RAEB-2, regardless of the blast percentage.

Patients with RAEB-2 have worse prognosis and a higher rate of transformation to AML. The median survival time is < 2 years in most studies, and although 30% to 40% of patients develop acute leukaemia, many die of the consequences of neutropenia, thrombocytopenia, or anaemia.(13) Median survival time for RAEB-1 is approximately 16 months vs 9 months for RAEB-2.(13) Approximately 25% patients diagnosed with RAEB-1 and 33% of patients with RAEB-2 progress to acute leukaemia.(1)

In about 15% cases of MDS, there is significant reticulin fibrosis in the bone marrow. These cases have been called MDS with fibrosis(MDS-F).(1)The diagnosis of MDS-F requires the presence of diffuse, coarse reticulin fibrosis with or without concomitant collagenisation, associated with dysplasia in atleast two cell lines.(1) Most of these cases with fibrosis belong to the RAEB category because of the presence of excess blasts which can be demonstrated using immunohistochemistry for CD34.(1) Marrow fibrosis can be present at the time of diagnosis or may develop during the course of the disease. Recently, the presence of marrow fibrosis has been recognised as an adverse prognostic factor in patients with primary myelodysplastic syndromes.(25)

MYELODYSPLASTIC SYNDROME WITH ISOLATED DEL (5q):

Also termed 5q deletion syndrome.

This subtype of MDS is characterized by anaemia with or without other cytopenia(s) and/or thrombocytosis and a sole cytogenetic abnormality of del (5q). Myeloblasts constitute less than 5% of nucleated bone marrow cells and less than 1% of peripheral blood leukocytes. Auer rods are not seen.

This syndrome is more common in women with a median age of 67 years. The proposed etiology is an interstitial deletion of the long arm of chromosome 5 which might have a possible tumour suppressor gene such as early growth response 1(EGR1) and a-catenin(CTNNA1).(1) Recently, a defect in ribosomal protein function due to haploinsufficiency of *RPS14* has been identified in the pathogenesis of the 5q– syndrome.(13)Patients with isolated del(5q) have a very favourable response to the drug Lenalidomide.(13)

MDS UNCLASSIFIABLE:

This category encompasses cases that do not fit easily into the other categories of MDS. Three possible situations that fit for this diagnosis include:

- Cases that have the criteria for a diagnosis RCUD or RCMD but with a percentage of 1% blasts in the blood, detected consistently in at least 2 occasions.
- MDS cases with morphologic unilineage dysplasia but associated with pancytopenia.

- Cases with persistent cytopenia(s) that lack the morphologic features of MDS (<10% dysplasia in any lineage) but with cytogenetic features with possible evidence of MDS.

Patients with this subtype should be followed up carefully for evidence of evolution to a more specific subtype of MDS.

The current revision of the WHO classification states "Cases with chromosomal abnormalities compatible with a myelodysplastic syndrome associated with strong clinical evidence of MDS but in which dysplastic features are less than 10% of cells in one/ more of the cell lineages are classified as MDS, unclassifiable.(13)

Occasionally, there are patients who have persistent cytopenia (usually anaemia) for which no underlying cause is found. They also have insufficient morphologic evidence to support the clinical possibility of an MDS. For some of these patients, a "working diagnosis" of idiopathic cytopenia of unknown significance may be considered. (1)(13)

It is important to understand that this category is not synonymous with a diagnosis of MDS.

CHILDHOOD MYELODYSPLASTIC SYNDROME:

Myelodysplastic syndromes are very rare in children. Both primary and secondary MDS can occur. The "secondary MDS" may follow congenital or acquired bone marrow failure disorders and also following cytotoxic therapy. Certain significant differences are observed between MDS in adults and those in children.

- Isolated anaemia, the common presentation in adults is uncommon in children. Neutropenia and thrombocytopenia are more common.
- Hypocellularity of the marrow is more commonly seen.

Because of these differences a new term Refractory cytopenia of childhood has been introduced.(1)

REFRACTORY CYTOPENIA OF CHILDHOOD (RCC):

This is defined as a myelodysplastic syndrome characterised by persistent cytopenia with <5% blasts in the bone marrow and <2% blasts in the peripheral smear.

RCC is the most common type of MDS in children constituting about 50% of the cases. It affects boys and girls equally. In addition to the dysplastic changes observed in bone marrow aspirate smears, examination of trephine biopsies is necessary for the diagnosis of RCC. The bone marrow cellularity may range from hypocellular to hypercellular, however majority of patients show a marked decrease in the marrow cellularity. Myeloblasts are typically less than 5%. Ring sideroblasts are not seen. Megakaryocytes may be normal, increased or decreased in number.

The detection of micromegakaryocytes is a strong indicator of RCC. The bone marrow reticulin content is normal. Most cases of RCC show a normal karyotype. Monosomy 7 is the most commonly observed karyotypic abnormality. Other complex karyotypes may also be found.

It is noteworthy to remember that morphologic dysplasia is not necessarily synonymous with myelodysplastic syndromes (MDS). There are other non clonal causes of dysplasia which should be excluded in the appropriate clinical setting. These include a number of pathological conditions.

Dysplastic features may be observed in a briskly regenerating marrow. Conditions causing stress erythropoiesis such as haemolytic anaemias may show features of dyserythropoiesis. In patients with drug/toxin induced or autoimmune related neutropenia, the myeloid cells show left shift in maturation and may demonstrate hypogranulation or may contain abnormally coarse granules. In ITP, there are many young megakaryocytes that are often monolobated and hypergranular.

Features of dysplasia are seen in inherited bone marrow failure syndromes. This may mimic MDS, especially in pediatric patients. Noonan syndrome, congenital dyserythropoietic anaemia, Pearson syndrome (mitochondrial disorders), Dobowitz syndrome, reticular dysgenesis, thrombocytopenia with absent radii (TAR) and amegakaryocytic thrombocytopenia can share some clinical and morphological features with MDS. These syndromes may not be necessarily associated with an increased risk of transformation to acute myeloid leukaemia.

In persons with congenital bone marrow failure syndromes, hematopoiesis can show dysplastic features, particularly in the erythroid lineage. Many bone marrow failure syndromes cannot be separated from MDS based on the presence or absence of dysplasia alone. To make the distinction, a thorough clinical examination, physical examination, family history and other laboratory tests need to be considered.(11)

In 29% to 45% cases of paediatric MDS, an associated constitutional abnormality is present. The development of MDS in such settings should be considered if hypercellularity of the bone marrow develops in the presence of peripheral cytopenia(s) or if a persistent clonal chromosomal abnormality is detected.

Table 3: Myelodysplastic syndromes – Differential Diagnosis

MDS	Distinguishing features
Peripheral destruction (haemolytic anaemia, ITP)	• Preserved bone marrow topography. • Hyperplasia of cytopenic lineage. • Increased or normal hematogones
Growth factor treatment	• Clinical history. • Minimal morphological dysplasia • Hypergranulation in neutrophils
Aplastic anaemia	• No increase in the CD34 + precursor cells by immunohistochemistry. • Mild dysplasia often in erythroid lineage.
Bone marrow failure syndromes (congenital)	• Clinical and family history. • No clonal cytogenetic abnormality
Hairy cell leukaemia	• CD20 +ve neoplastic B cells in the bone marrow biopsy.

ROLE OF CYTOGENETICS IN THE DIAGNOSIS OF MDS:

Cytogenetic profile has become a standard practice in the diagnosis of myelodysplastic syndromes. It is a major indicator for predicting clinical course and outcome.(7)

Clonal cytogenetic abnormalities are noted in approximately 50% cases of primary or denovo MDS and upto 80% of myelodysplastic syndromes that develop secondary to chemotherapy or other toxic agents. (26)

Table 4: Chromosomal abnormalities in myelodysplastic syndrome (MDS)

UNBALANCED	**BALANCED**
+8	t(11;16)(q23;p13.3)
del(7q)	t(3;21)(q26.2;q22.1)
del(5q)	t(1;3)(p36.3;q21.2)
del(20q)	t(2;11)(p21;q23)
-Y	t(6;9)(p23;q34)
t(17p)	Inv(3)(q21;q26.2)
del(13q)	

Conventional karyotyping is an essential component of the diagnostic work up of any patient suspected to have MDS. They form the basis for the selection of drugs for individual cases of MDS. Myelodysplastic syndromes show characteristic genetic profile, the majority of which are unbalanced translocations. Unlike in cases of acute myeloid leukaemia, cases of MDS show partial or total chromosomal losses, high incidence of chromosomal gains and rare translocations. But, the results have to be interpreted with caution. The presence of these cytogenetic abnormalities as the sole finding in the absence of the morphological features is not considered as a definite evidence for MDS. (1)

In order to avoid false positive results, a standard definition for clonality such as the presence of same chromosomal gain or structural aberration in at least two bone marrow cells and the same chromosomal loss in at least three bone marrow cells is essential.

In a study by Chaubey *et al,* on the cytogenetic profile of myelodysplastic syndrome in 50 Indian patients, the most common cytogenetic abnormality was Monosomy 7. The other abnormalities were del (5q), +8, del (6q) and del (3q) in the decreasing order of frequency. Del (6q) and del (3q) were two new cytogenetic abnormalities that were not described so far. They found clonal cytogenetic abnormality in 47.5% of cases which is comparable to the published data so far.(7)

The loss of 17p is associated with MDS or AML with pseudo-Pelger Huet anomaly, TP53 mutations, small vacuolated neutrophils and an unfavourable clinical outcome.(1) Complex karyotypes which include >3 cytogenetic alterations typically include chromosome 5 and/or 7 and are generally associated with an unfavourable clinical course.(1)

Isolated del(20q) is associated with morphological abnormalities involving the erythroid and megakaryocytic lineages.(1)Inversion (3) is a rare cytogenetic abnormality in MDS. It is also noted in cases of AML and blast phase of chronic myeloid leukaemia. The bone marrow biopsies of cases of MDS with inv (3) are usually normocellular or hypercellular and show megakaryocytic hyperplasia. The megakaryocytes have a monolobated or hypolobated nuclei. The blast count ranges from 0 to 10%. MDS cases associated with inv (3) have a poor clinical outcome, resistant to chemotherapy and have a higher risk of transformation to acute myeloid leukaemia.(27)

The IPSS uses cytogenetic abnormalities to stratify cases of MDS into three different risk categories, namely good, poor and intermediate.(11)

Good: Includes normal karyotype, isolated interstitial del (5q), isolated del (20q) and –Y.

Poor: Includes cases with complex karyotype, del (7q) and -7

Intermediate: Includes all other abnormalities.

IPSS prognostic subgroup, proportion of the marrow blasts and haemoglobin level was found to be the main prognostic factors for survival. (28) The first two factors and the platelet count were the best predictors for risk of transformation to acute leukaemia.(28)

ROLE OF FLOWCYTOMETRY:

Immunophenotyping using flowcytometry is not routinely used in the diagnosis of MDS. But, flowcytometric immunophenotyping has been shown to correlate with cytogenetic abnormalities and morphological dysplasia. The objective of flowcytometry is to identify dysregulated antigen expression in the neoplastic cells. The advantage of flowcytometry is that, it is less subjective, more sensitive and less affected by the quality of specimen when compared to the morphological assessment of dysplasia.(29)(11) Flowcytometric abnormalities alone are not sufficient to arrive at a diagnosis of MDS .(1)

The diagnosis of MDS by flowcytometry is based on the interpretation of altered patterns of maturation and myelomonocytic differentiation, by using combination of antigens such as CD11b/CD16, CD33/HLA-DR, CD13/CD16, CD64/CD10. There are certain limitations to this method. Certain conditions such as a regenerating marrow, growth factor therapy, severe infection, acute bone marrow insults, HIV and autoimmune disorders also might show alterations in the myelomonocytic maturation patterns by flowcytometry. Abnormal expression of some markers such as a decreased CD33 expression can be due to genetic polymorphisms and not necessarily due to dysplasia.

The other approach in the diagnosis of MDS is by focusing on the phenotype of myeloblasts. In a reactive marrow, CD34+ve cells show diverse differentiation and maturation patterns. Normally, CD34+ve stem cells are capable of producing hematogones (CD19+ve and CD10+

immature B-cells precursors), myeloid precursors (CD34+ve, CD65+ve, CD15+ve), monocytic precursors (CD34+ve, CD4+ve, CD64+ve) and plasmacytoid dendritic cell precursors (CD123+ve(bright), HLA-DR+ve). In MDS, the CD34+ve blasts are clonal forming a discrete population without evidence of differentiation towards monocytes, hematogones or plasmacytoid dendritic cells. Aberrant expression of lymphoid antigens such as CD2, CD7, CD5 and CD56 can also be noted.

A positive flowcytometry result does not help to distinguish MDS from MDS/MPN or from MPN. Chronic idiopathic myelofibrosis can show similar changes like MDS. The better approach for cases with borderline features of dysplasia and no cytogenetic abnormalities showing features of MDS in flowcytometry, would be to perform a re-evaluation after several months to look for morphological and cytogenetic features of MDS.

Flowcytometry is not a recommended method for enumeration of the percentage of blasts.All the myeloblasts donot express CD34 and cells such as early hematogones can also express CD34. Hence, flowcytometry underestimates the percentage of blasts. Morphological counting or immunohistochemistry done on the trephine biopsy sections are more useful for the assessment of blast percentage.

ROLE OF FLUORESCENCE IN SITU HYBRIDIZATION (FISH) IN MDS:

In MDS, FISH has been used to identify recurring genetic abnormalities. The panel include probes for del (5q), del (7q), trisomy 8 and del (20q).FISH can be helpfulin cases of MDS with karyotype failure, in cases of RAEB and RCMD with a normal G-band karyotype. The usefulness is limited in cases of RCUD and in cases with an abnormal karyotype by

conventional karyotyping. The advantage of FISH is its high sensitivity due to the number of scorable cells compared with routine analysis of only 20 cells by cytogenetics.

Comparative genomic hybridization arrays, single-nucleotide Polymorphism arrays and molecular testing are other tools that have been found to be useful in the diagnosis of MDS, but are not used in routine practice.

ANGIOGENESIS IN MYELODYSPLASTIC SYNDROME:

Angiogenesis or neovascularisation is formation of new blood vessels from pre-existing vessels. The process of angiogenesis can occur by two mechanisms. The first mechanism is by branching and extensions from pre-existing vessels. The second mechanism is by the recruitment of endothelial progenitor cells to the site of formation of the new vessel. (30)

Angiogenesis is a basic process that affects day to day physiological reactions such as wound healing, regeneration, menstruation etc. Angiogenesis also plays a prominent role in pathological processes such a development of tumours and metastasis, chronic inflammation and conditions such as diabetic retinopathy.

The process of formation of new vessel from a pre-existing vessel involves vasodilation, proteolytic degradation of the basement membrane of the parent vessel, movement and proliferation of the endothelial cells towards the angiogenic stimuli forming capillary sprouts, maturation of endothelial cells and stabilisation of the new vessel by recruitment of periendothelial cells.(30)

Neovascularisation also occurs by the mobilisation and recruitment of endothelial progenitor cells from the bone marrow to the site of injury or tumour. These endothelial progenitor cells also express some markers of hematopoietic stem cells, VEGFR-2 and VE-Cadherin.

Angiogenesis is controlled by a balance between angiogenesis inhibitors(angiostatin, endostatin and vasculostatin) and promoters(VEGF and bFGF). The angiogenic switch is activated either by a loss of angiogenic inhibitors or increased production of angiogenic promoters. The angiogenic switch is also upregulated by hypoxia. The lack of oxygen in the cell environment stimulates production of HIF1a(Hypoxia induced factor 1a) which upregulates the promoters of angiogenesis such as VEGF thus creating a proangiogenic environment. (30)

VASCULAR ANATOMY OF THE BONE MARROW:

The bone marrow can be subdivided into a hematopoietic compartment and a stromal compartment which is constituted by fibroblasts, adipocytes, nerves and the vascular system. (31)

The arterial vessels enter the marrow through the nutrient foramen and branches into several arterioles. Smaller arterioles and capillaries arise from these vessels, span the entire marrow space and feed the sinusoids. The sinusoids are interconnected by capillaries. These sinusoids are located around a central draining sinus which measures about 100microns in diameter. The bone marrow sinusoids are formed by a single layer of endothelial cells with no supporting structures. (32) The surrounding hematopoietic tissue appears to be the major cellular support for the sinusoidal microcirculation.(31)

Angiogenesis has been shown to affect the pathogenesis and prognosis in patients with myelodysplatic syndrome. A study performed by Savic *et al*, by investigating 70 patients with MDS showed an increased microvessel density (MVD) in patients with MDS. However, they found that angiogenesis was not an independent prognostic factor in patients with MDS. (33)

By assessing the angiogenesis in 82 patients with MDS and comparing it with 14 controls, five cases with infectious disease, 15 cases with AML and 14 patients with MPD, Pruneri *et al*, demonstrated that neovascularisation in cases of MDS was intermediate between those of controls and AML. They suggested that increased MVD could correlate with transformation of MDS into leukaemia. (34)

In the above study, the investigators used anti CD34 antibody and anti CD31 antibody to evaluate the degree of angiogenesis/ neovascularisation. They have shown that anti CD34 antibody is superior to anti CD31 antibody for assessing the MVD. The anti CD34 immunostain highlights variable number of blasts and endothelial cells in the bone marrow. There was good inter observer reproducibility when anti CD34 immunostainwas used to assess the degree of angiogenesis. The MVD intra reader variability was found to be 14 ± 10%*(r* = 0.879).

On the contrary, anti CD31 antibody also stained myeloid cells, lymphoid cells and megakaryocytes, in addition to the endothelial cells, thus resulting in high amount of nonspecific back ground staining.(34) However, it becomes necessary to note that, when using the anti CD34 antibody, enumeration of the vessel density often becomes difficult when there is a high blast percentage obscuring the microvessels.

Different investigators have made use of different guidelines and methods to quantitate the microvessel in the bone marrow. The most commonly used method is the “hotspot method”. In this method, after immunostaining with the antibody, the entire section is initially screened under low power magnification (40x) and 5 or 10 spots with the highest amount of microvessels are marked. These five fields, termed the microvessel hotspots are examined under the high power magnification (400x) and the number of vessels per high power field is

counted by a minimum of two observers to reduce the inter-observer variability. The final count on the degree of microvessel density of the entire marrow is given as the average count of microvessels in these five fields or the five hotspots. Several studies have reported the MVD by the above mentioned "hotspot" method for assessing microvessel density not only in cases of MDS but also in other haematological malignancies such a multiple myeloma and chronic lymphocytic leukaemia. (35)(36)

Similarly, Lundberg *et al* demonstrated a higher degree of microvessel density in patients with MDS compared to healthy controls. In this study, the degree of angiogenesis neither correlated between various subgroups of MDS nor with the overall survival of the patients.(37)

SCREENING FOR MYELODYSPLASTIC SYNDROME THROUGH THE AUTOMATED CELL ANALYSER:

The diagnosis of myelodysplatic syndrome (MDS) requires a high index of suspicion by the clinician. The cause of peripheral cytopenia(s) may be due to myriad haematological and non haematological factors. Although there are a number of previously described and documented morphological features of dysplasia, the assessment of these features in the peripheral blood smears and bone marrow aspirate smears is subjective and is labour intensive.

Evaluation of the dysplastic features in the light microscopy in peripheral blood films is influenced by a variety of factors such as the pH of the stain, quality of staining, the time interval between collection and preparation of the smears, interobserver variation and morphological alterations due to secondary causes such as drugs and storage artefacts.

Automated haematology analysers such as Sysmex, Beckman Coulter system etc.have become an integral part of any modern laboratory. The result obtained from these analysers in routine laboratory setting is the complete blood count (CBC) which gives a quantitative assessment of the Haemoglobin, total leucocyte count, differential leukocytecounts and the platelet count.

The automated haematology analysers use various analytical principles to quantitate blood cells in a peripheral blood sample and to perform a differential count. The principle used by these analysers is known as the Coulter Principle.

COULTER PRINCIPLE:

A suspension of blood cells is passed through a small orifice simultaneously in the presence of an electric current. The individual blood cells passing through the orifice introduce an impedance change in this orifice which reflects the size and the number of cells.

The Coulter method accurately enumerates and sizes the cells by detecting and measuring changes in the electrical impedance when a cell passes through the orifice or a small aperture. Particles such as blood cells are nonconductive but are suspended in an electrically conductive diluent. As each cell passes through the aperture, it momentarily increases the resistance of the electrical field between the submerged electrodes located on either side of the aperture. This is recorded as an electronic pulse. The number of pulses indicate particle count and the size of the electrical pulse is proportional to the cell volume. This is based on the principle of impedance counting.

This mechanism of impedance counting is useful to separate the major groups of cell lines such as erythrocytes, leukocytes and platelets. The estimation of the number and the volume is also possible based on the Coulter principle. For further sub classification of white blood

cells into neutrophils, eosinophils, basophils, monocytes and lymphocytes, the internal structure of these cells need to be accessed as they differ in their cytoplasmic granule content and nuclear complexity.

For the purpose of measuring the internal complexity of cells the ability of these cells to scatter light was exploited. A focused stream of cells is interrogated by a laser beam directed against them. The cells in turn scatter light depending on their surface and their internal structure. The scattered light is in turn measured to assess the cytoplasmic content and nuclear complexity by the principle of flowcytometric counting.

Modern haematology analysers such as Beckmann Coulter DxH800, incorporate the above mentioned method of impedance counting and flow cytometric counting through the VCS technology.

VCS TECHNOLOGY:

A diluted blood sample is passed in a steady stream onto which a beam of laser light is focused. The WBC differential count is established by the automated cell counters using three measurements: the individual cell volume, conductivity and the light scatter by the individual cells.

Volume: The leukocyte volume analysis is performed using the low frequency current. This is based on the principle of impedance counting as described above.(Page 38)

Conductivity: Cell walls of each cell act as conductors to high frequency current. The current detects differences in the insulating properties of the cell components, while passing through the cell walls and through the interior of the cell. The current characterises the granular and nuclear constituents and the chemical composition of the interior of the cell.

Light scatter: When a cell is struck by the coherent light of a laser beam, the light scatters and spreads out in all directions. Using detectors, the light scatter signals are collected to get information regarding the granularity of the cell, nuclear lobularity and the cell surface. The scattered light gives information regarding the cell structure, shape and the surface. Previous automated analysers such as LH 750 and 755 used only a single scatter measurement. The UniCel DxH 800 cell counters have improved differential accuracy and efficiency of flagging by incorporating additional light scatter measurements such as UMALS, LMALS, LALS and AL. This information is utilised to produce a scatter plot with a five part differential count.

Out of the five different scatter measurements, LMALS is a side scatter and is considered to be a reliable indicator of the cell content and granularity. The data obtained by the cell counters using the VCS technology is known as the Cell Population Data (CPD data). This CPD data is available in the instrument as separate values for volume, conductivity and scatter for each of the four major white blood cells which include neutrophils, lymphocytes, monocytes and basophils. The instrument utilises this information to calculate and provide a consolidated final WBC differential count.

In addition to the peripheral leukocyte counts, these automated haematology analysers also show specific identifiable patters of cell population and distribution in the form of histograms and scatter plots. These patterns show specific alterations in various pathological conditions such as bacterial infections (38)(39)(40), malaria(41), chronic lymphoproliferative disorders (42), myocardial infarction(43) and few others. These alterations have been shown to reflect the cellular changes seen in the light microscopy.

For example, Lee *et al* demonstrated that mean cell volumes of neutrophils and monocytes can be used to identify patients with sepsis. Using Coulter LH750, they studied the mean

volumes of neutrophils and monocytes in 18 patients with sepsis, 38 patients with localised infections and 29 healthy controls. The mean neutrophil volume and the mean monocyte volume were found to be significantly higher (p-value = 0.000 for both) in patients with sepsis compared to those with localised infections and healthy controls. The mean cell conductivity and the lower median angle light scatter were significantly lower in the group with sepsis in comparison with those with localised infections and healthy controls (P-value 0.029 and 0.022 respectively).(44)

Several studies are being done at present, to determine if data from the automated haematology analysers can be reliably used as a screening or a diagnostic tool for the identification of myelodysplastic syndrome (MDS) in the routine clinical setting. (45)

Initial attempts in the identification of cases of MDS through the peripheral blood samples by an automated haematology analyser were performed as early as 1987 when Rappaport et al, reported differences in the cell counter values in 5 cases of MDS when compared to the normal individuals. (46)

.A research paper published in the journal of clinical chemistry and laboratory medicine by Elisabeth *et al*, in 2008 showed that research population data from the automated cell counter could be routinely used to screen for myelodysplastic syndrome and chronic lymphatic leukaemia.(47)In this study, blood samples from 44 cases of chronic lymphatic leukaemia, 19 cases of myelodysplatic syndrome and 199 healthy blood donors were analysed using the Beckman Coulter LH750.

The mean and standard deviations of the volume, conductivity and scatter of the neutrophils were analysed in the 19 cases of MDS and all the healthy controls. The research population data turned out to be significantly different among the groups. The standard deviations of the

neutrophil conductivity was found to be predictive for myelodysplastic syndromes. (sensitivity 88.6%, specificity 84.4%).(47)

The role of two neutrophil parameters namely, NEUT-X and NEUT-Y (Sysmex XE-2100) in the diagnosis of MDS was studied by Goel *et al*. NEUT-X is the measurement of side scatter diffraction and represents the internal structure of the neutrophils. This correlates with the hypogranularity of the neutrophils which is seen in MDS. This study included four groups, namely: 1. Controls 2. MDS 3. MPN 4. Megaloblastic anaemia. The mean value of NEUT-X was found to be significantly lower in MDS compared to the controls. On the contrary, the mean value of NEUT-X was significantly higher in cases of megaloblastic anaemia.(48)

A recent study by Raess *et al* demonstrated that cases of MDS have significantly decreased haemoglobin, haematocrit, WBC count and platelet counts compared to normal controls. In addition, patients with MDS had an elevated MCV, MCH, RDW and PDW compared to the normal controls. (45) Automated blood counts showed decreased percentage of neutrophils and corresponding increase in the percentage of the lymphocytes in cases of MDS.

The differences in individual CPD parameters for neutrophils, lymphocytes and monocytes between MDS and control cases were not significant. But, mean conductivity showed lower values in cases with MDS, suggesting a possibility of differences in the internal composition of leucocytes. The standard deviation of neutrophil volume also had a trend towards being elevated in cases of MDS. (45)

In addition, Raess et al have developed a random forest classifier method that will help in the identification of patients having MDS with relatively high sensitivity and specificity. They proposed the applicability of this random forest classifier to discriminate cases of MDS from non MDS patients. The sensitivity of the classifier was found to be 85% and the specificity was 87.1%. By using the classifier, the most discriminating factors between the controls and

the MDS patients were mean neutrophil conductivity, mean neutrophil volume and the standard deviation of the neutrophil volume. In the standard conditions, the automated cell analyser flagged only 25% cases of MDS for subsequent peripheral blood examination, whereas the random forrest classifier identified 85% of patients with MDS.

Further prospective studies to evaluate the importance of automated haematology analyser is necessary before it can be used on a routine basis for the identification of MDS in current situation.

THERAPY RELATED MYELODYSPLASTIC SYNDROME:

This form of myelodysplastic syndrome (t-MDS) occurs as a late complication of prior administration of cytotoxic drugs and radiation therapy for haematological or non-haematological neoplasms.

This category of MDS is considered under the broad heading of therapy related myeloid neoplasms by the WHO classification. The other disorders that are present under these group of neoplasms are t-MDS/MPN and t-AML. These three categories are diagnosed based upon the degree of dysplasia and the number of blasts. However, all of these therapy related neoplasms are best considered a single clinical entity. (1)

The common cytotoxic agents implicated in therapy related myeloid neoplasms are Alkylating agents (melphalan, cyclophosphamide, busulfan), Topoisomerase type II inhibitors (Etoposide, doxorubicin, daunorubicin), antimetabolites, antitubulin drugs (vincristine, vinblastine) and ionising radiation therapy.

Any age group may be affected. Generally, two subsets of patients are recognised. The first being t-MDS or t-MDS/t-MPN who present with bone marrow failure and peripheral

cytopenia(s). The second subset being t-AML/t-MDS or t-AML/t-MDS/t-MPN. The second subset of patients comprise about 20 to 30% cases and present with an overt leukaemia without the prior myelodysplastic phase with a very short or no myelodysplastic phase.

The majority of patients have multilineage dysplasia. Anaemia is almost always present with evidence of macrocytosis and poikilocytosis in the peripheral smear. Neutrophils particularly show nuclear hypolobation and cytoplasmic hypogranularity. Dysmegakaryopoiesis is also seen in the form of pawn ball megakaryocytes, mono or hypolobated forms. Almost 50% of the patients have lesser than 5% blasts in the bone marrow.

Approximately 70% of patients with t-MDS harbour unbalanced chromosomal aberrations involving chromosomes 5 and 7. The prognosis of t-MDS is generally poor. The survival is strongly influenced by the nature of the karyotypic abnormality and the underlying malignancy for which the cytotoxic therapy was administered.

MANAGEMENT:

The treatment for MDS is aimed at controlling symptoms, improving the quality of life and to prevent the progression of disease to leukaemia. Three agents have been approved by the FDA in the treatment of MDS, namely 5-azacytidine, Decitabine and Lenalidomide. The latter is used only in the treatment of 5q deletion syndrome. Stem cell transplantation particularly in younger patients offers the potential for curative therapy.

Red cell transfusions are a major part of supportive therapy for patients with anaemia. Due to the repeated transfusions, patients develop iron overload and its metabolic consequences. Iron chelators are used to overcome this problem.

Drugs which inhibit angiogenesis have also been found to be useful in the treatment of MDS. As mentioned above, studies have documented the presence of an enhanced degree of MVD in the bone marrow microenvironment of patients with MDS. Generally, endothelial cells are considered to be more genomically stable compared to the genetically unstable cancer cells. Here lies the advantage of targeting endothelial cells instead of the cancer cells which rapidly acquire new mutations that confer rapid drug resistance to the conventional chemotherapeutic drugs.

In summary, myelodysplastic syndromes are clonal hematopoietic stem cell neoplasms with a high propensity to transform into acute leukaemia. Patients with MDS almost always present with clinical symptoms manifested as a consequence of peripheral blood cytopenia(s). The peripheral cytopenia(s) are due to apoptosisof the marrow progenitor cells resulting in ineffective haematopoiesis. MDS can be a primary denovo disease or secondary due to cytotoxic drugs. The peripheral blood smear and bone marrow show several pathological cellular and stromal alterations. The cellular alterations manifests as dysplasia in the bone marrow progenitor cells and is reflected in the peripheral blood as cytopenia and abnormal cell morphology. The stromal alterations is usually in the form of increased reticulin fibrosis. MDS has been further subtyped based on their clinical, morphological, immunological and cytogenetic profiles by the WHO. IPSS score is used by the clinician to stratify patients into three risk categories. Several studies are being done at the cellular and molecular levels to gain a better understanding of the complex pathological alteration of these group of disorders.

RESEARCH AIMS AND OBJECTIVES

THE RESEARCH AIMS:

1. To study the cellular alterations in cases of MDS.

2. To study the stromal vascular changes in cases of MDS.

THE RESEARCH OBJECTIVES:

1. To evaluate the role of cell population data (CPD) values obtained through the automated haematology cell counter (Beckman Coulter DxH800) in the diagnosis ofMDS .

2. To look for differences in the CPD values among the MDS cases, non MDS cases and healthy controls.

3. To enumerate the Microvessel Density (MVD) in the bone marrow trephine biopsy of patients with MDS, using anti CD34 antibody.

4. To compare the values of MVD between MDS cases and controls.

MATERIALS AND METHODS

METHODOLOGY:

This study was approved by the Institutional review board of the Christian Medical College, Vellore.

The study spanned a period of 14 months from the 1st of May 2013 to the 30th of June 2014

EPIDEMIOLOGICAL METHOD: Analytical epidemiology – Comparative study.

SELECTION OF CASES:

- **Diagnostic criteria**: All the cases of primary (denovo) MDS diagnosed in the study period. The diagnosis was based upon clinical data, routine peripheral blood counts, examination of peripheral blood film, bone marrow study and cytogenetic analysis.

- **Eligibility criteria**: Only the newly diagnosed cases in the specified study period were included. Old cases of MDS on follow up and cases of secondary MDS were excluded.There were a total of 124 cases of MDS diagnosed in the Christian Medical College and Hospital during this period. Out of the 124 cases of MDS recorded, 111 were primary (denovo) MDS and 13 cases were either on treatment or therapy related. Consequently, only the data obtained from the 111 cases of primary (denovo) MDS are discussed below.

Sources of the cases: All the 111 cases included in the study were patients who visited the Christian Medical College Hospital in the specified study period.

SELECTION OF CONTROLS:

As the study had two aims, the numbers and character of the controls were selected separately according to the objective.

For the purpose of assessment of the CPD, two sets of controls were used in the study. One set consisted of 155 patients with illnesses other than MDS. These 155 non MDS controls were age matched and selected from the same source as the cases. The second set of controlswere 98 healthy blood donors with no present illness. The collection and processing of the peripheral blood samples of both the controls and cases were identical.

For the purpose of assessment of the MVD in the trephine biopsies, 35 age matched controls were chosen. These controls were patients who had undergone bone marrow biopsies as a part of staging for other haematological and non haematological malignancies and were found to be free of marrow involvement.

DATA COLLECTION:

These necessary study parameters and data for these 124 cases were retrospectively collected from the department archives.

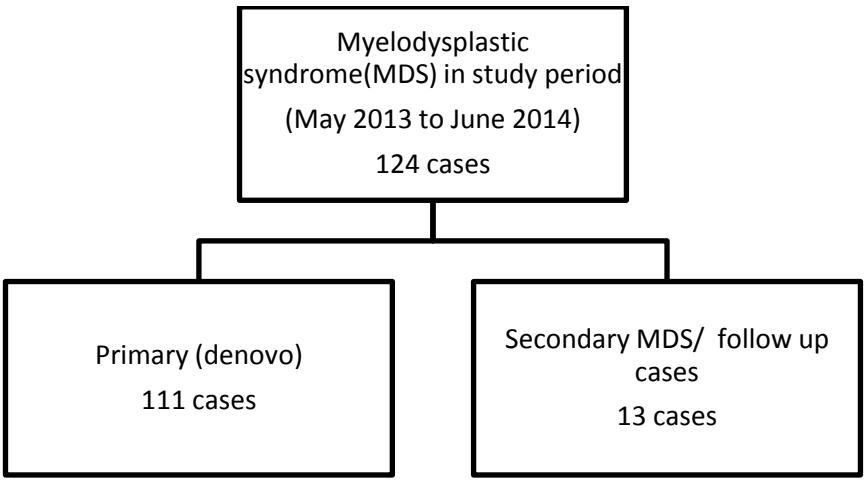

Figure 4: Distribution of cases in the study period(May 2013 to June 2014).

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The demographic and the clinical information of the cases were collected from the electronic medical records.

Out of the 111 cases included in the study, the CPD values were available for 68 cases. These 68 cases were compared with 155 non MDS samples and blood samples from 100 healthy blood donors. The non MDS group consisted of those cases, who were being treated in various departments in the hospital as an outpatient or as in patient and did not have peripheral blood cytopenia(s) or other diagnosed haematological disorders.

The 155 samples from the non MDS cases and 100 cases from normal healthy blood donors were processed in the same manner as followed for the MDS samples.

Secondly, out of the 111 cases, the paraffin embedded tissue blocks were available for 107 cases. The originally stained slides at the time of diagnosis and the blocks were collected from the archives. Immunohistochemistry was performed on the paraffin embedded blocks with anti CD34 antibody.

COLLECTION OF THE CELL POPULATION DATA:

The CPD parameters for the cases were collected from the Beckman Coulter DxH800 automated haematology analyser. This is a high volume, fully automated haematology analyser which is capable of analysing upto 100 sample/ hour. The analyser makes use of the Coulter principle, optical detection and radiofrequency conductivity for cell counts and differential counts. This analyser provides a CBC, 5 part WBC differential count, reticulocyte count and NRBC counts from 165uL of blood sample.

The specimens were peripheral blood samples collected with dipotassium EDTA as anticoagulant. The samples were placed in the room temperature and processed within 2 hours of collection from the patient, thereby excluding any effects of delayed processing.

The CPD data were retrospectively collected form the MDS cases after a diagnosis of MDS was made by the examination of the peripheral blood film and the bone marrow. Only the first sample from each patient during the study period was used for the analysis in patients who had frequent CBCs.

The CPD parameters that were compared between the three groups include the neutrophil volume, neutrophil conductivity, lymphocyte volume, lymphocyte conductivity, monocyte volume, monocyte conductivity, eosinophil volume, eosinophil conductivity. Lower medial angle light scatter (LMALS) for neutrophils, lymphocytes, monocytes and eosinophils.These parameters were periodically checked as a part of routine QC program and maintained within acceptable limits using Levey-Jennings charts and appropriate Westgard rules.

IMMUNOHISTOCHEMISTRY IN THE BONE MARROW TREPHINE BIOPSY:

ANTI-CD34 MONOCLONAL ANTIBODY:

The antibody used was anti-CD34 monoclonal mouse IgG1 antibody. This antibody recognises a glycosylated type I 110-kDa transmembrane molecule which is considered to be a marker for the blood vessel endothelium.

The sections were deparaffinised and rehydrated in graded series of alcohol. The sections were then labelled using an automated immunostaining procedure (Ventana medical systems). The antigen retrieval was performed by boiling at 95 to 100 C. The mouse

monoclonal anti-CD34 (Dako, clone 96 3820 B; 1:10 dilution) was incubated for 60 minutes at 42 C. Followed by this, the sections were then incubated with the universal anti-mouse Ig biotinylated secondary antibody for 16 minutes at 37C.

This was followed by incubation with the avidin-biotin-horseradish peroxidase (HRP) complex with the Ventana Medical Systems DAB detection kit for 8 min at 37C. The final revelation system used HRP-DAB as the final chromogen. Positive controls were bone marrow sections from patients with acute leukaemia. The slides were counterstained with hematoxylin.

ENUMERATION OF THE MICROVESSEL DENSITY (MVD):

Enumeration of the MVD in the bone marrow trephine biopsy sections was performed using the "hotspot" method. The bone marrow sections immunostained with anti-CD34 antibody was initially screened with the low power objective (4x) to identify hotspots which contained the maximum number of vessels. Five such hotspots were chosen by scanning the marrow section in the low scanning magnification (4x). These five areas or "hotspots" with the maximum microvessel density were thenexamined with the high power objective (40x). The number of vessels within each of these five hotspots with maximum density were counted. The MVD value of the particular marrow sample was given as the mean MVD of the five counted fields.

The hotspots were identified by two observers individually. If different hotspots were identified, the observers had to decide on which one was used for assessing the MVD in the five consecutive fields.

STATISTICAL METHODS:

- Data was analysed using the statistical package for Social sciences (SPSS) software, Windows version 16.

- Association of the CPD values between the different sub groups (MDS, non MDS, healthy donors) was calculated using the analysis of variance (ANOVA).

- Association of the bone marrow MVD between the MDS cases and controls was calculated using the Independent sample t-test.

- P values < 0.05 were considered significant.

- Sensitivity and specificity values were calculated using the ROC curves.

DATA ANALYSIS

ANALYSIS OF DATA:

DEMOGRAPHICS:

A total number of 111 caseswere included in the study.

Out of the total 111 patients, there were 74 (66.6%) malesand 37 (33.3%) females.

The youngest patient was 3 years old and the oldest was 78 years old with a median patient age of58.5 years.

Table 5: Distribution of cases by gender.

Gender	Number
Male	74 (66.6%)
Female	37(33.3%)
Total	111 (100%)

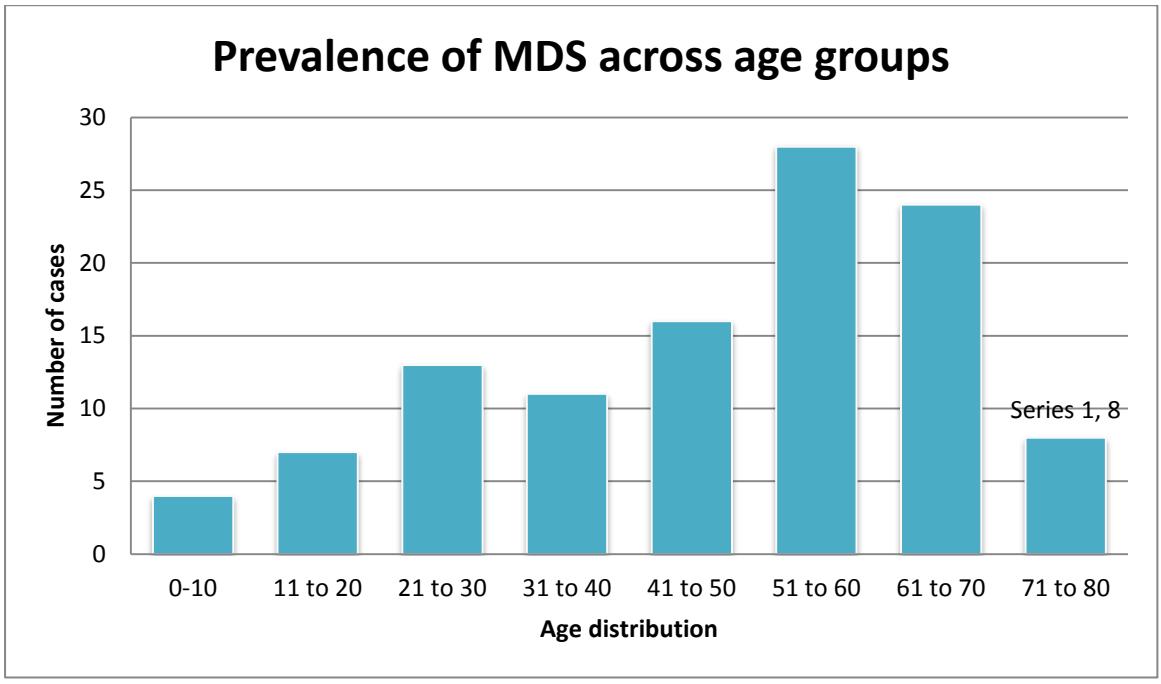

Figure 5: Age wise distribution of MDS cases.

Table 6: Distribution of cases according to the WHO subtypes.

WHO category	**Number of cases.**
Refractory anaemia with excess blasts-I	19 (17.1%)
Refractory anaemia with excess blasts-II	21 (18.9%)
MDS-F	2(1.8%)
Refractory cytopenia with multilineage dysplasia	4(3.6%)
Refractory cytopenia with unilineage dysplasia	34(30.6%)
Isolated 5q deletion syndrome	6(5.4%)
Refractory anaemia with ringed sideroblasts	6(5.4%)
MDS-Unclassifiable	7(6.3%)
Childhood myelodysplastic syndrome	2(18%)

The 111 cases of MDS were classified into low grade and high grade based on the blast percentage for the purpose of further analysis.

Bone marrow blast count of < 5% was taken as low grade and 5 or more was considered high grade. There were 44(39.6%) cases of high grade MDS and 67 (60.3%) cases of low grade MDS.

Table 7: Distribution of cases by grade.

Category of MDS	**Number of cases**
Low grade	67 (60.3%)
High grade	44 (39.6%)
Total	111 cases (100%)

COMPARISON OF CELL POPULATION DATA AMONG MDS CASES, NON MDS AND NORMALCONTROLS (HEALTHY BLLOD DONORS):

Among the 111 cases included, the cell population data from the automated cell counter was available for 68 cases.

Consequently, the data of these 68 cases was compared with 155 non MDS samples and with 98 healthy blood donors.

The CPD parameters of neutrophils, lymphocytes, monocytes and eosinophils were separately compared among the three groups.

Table 8: Comparison of the CPD parameters for neutrophils among MDS and non MDS patients:

NEUTROPHILS	**MDS cases (n-68)**	**NON MDS cases (n-155)**	**p-value**
	Mean(SD)	Mean(SD)	
VOLUME	153.76(16.44)	147.08(5.93)	0.000
CONDUCTIVITY	144.74(6.32)	147.68(4.87)	0.000
SCATTER	125.28(14.42)	136.94(6.19)	0.000

*p value is significant when <0.05

Table 9: Comparison of the CPD parameters for neutrophils among MDS and healthy donors.

NEUTROPHILS	**MDS cases (n-68)**	**Donors (n-98)**	**p-value**
	Mean(SD)	Mean(SD)	
VOLUME	153.76(16.44)	148.90(5.32)	0.002
CONDUCTIVITY	144.74(6.32)	145.61(2.46)	0.704
SCATTER	125.28(14.42)	132.88(5.87)	0.000

*p value is significant when <0.05

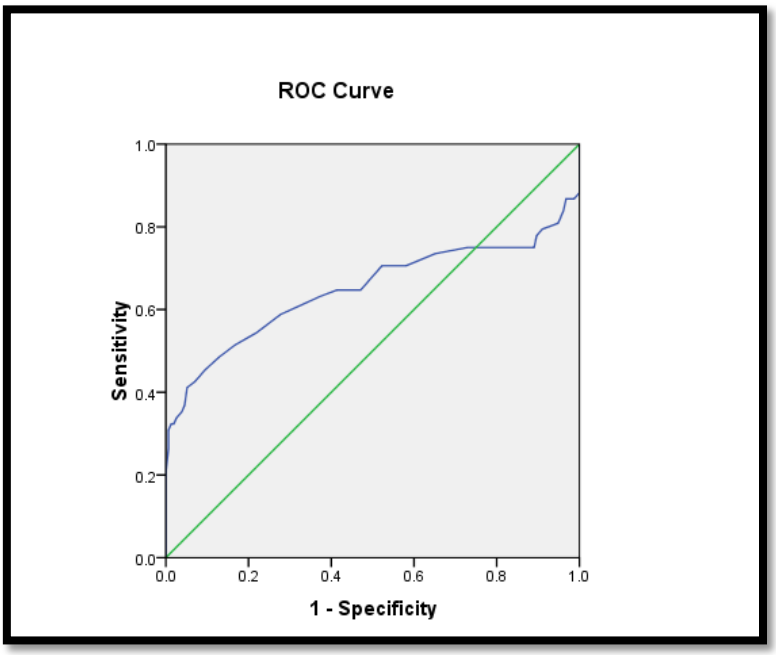

Figure 6: ROC curve for neutrophil volume.

By assigning the cut off value for mean neutrophil volume as 146.50, the sensitivity was found to be 70.6% and the specificity was 47.7%.

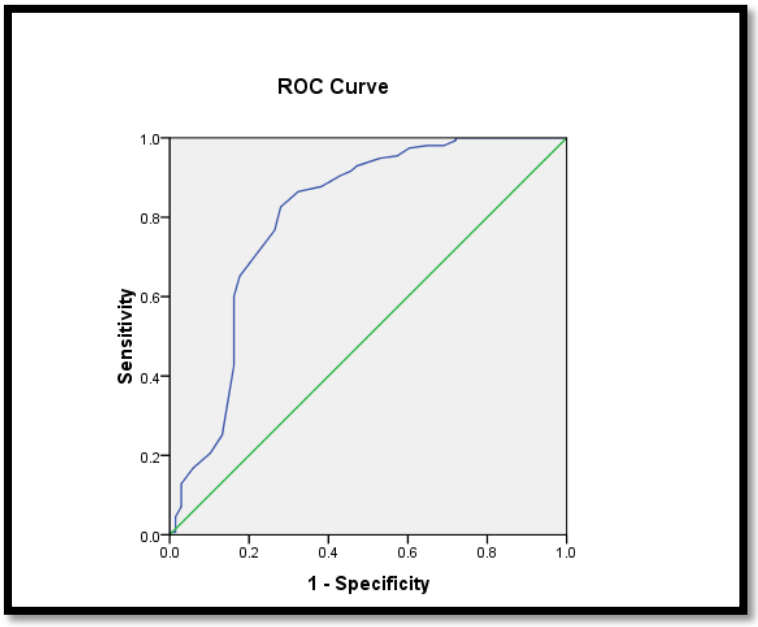

Figure 7: ROC curve for neutrophil scatter.

By assigning the cut off value for mean neutrophil scatter as 129.50, the sensitivity was found to be 90.3% and the specificity was 57.4%.

Table 10: Comparison of the CPD parameters for lymphocytes among MDS and non MDS patients:

LYMPHOCYTES	**MDS cases (n-68)**	**NON MDS cases (N-155)**	**P value**
	Mean(SD)	Mean(SD)	
VOLUME	90.84(8.70)	86.24(4.17)	0.000
CONDUCTIVITY	116.93(7.20)	117.37(4.23)	1.000
SCATTER	59.59(9.30)	60.83(4.59)	0.358

*p value is significant when <0.05

Table 11: Comparison of the CPD parameters for lymphocytes among MDS and non MDS patients:

LYMPHOCYTES	**MDS cases (n-68)**	**Donors (N-98)**	**p-value**
	Mean(SD)	Mean(SD)	
VOLUME	90.84(8.70)	84.57(2.81)	0.000
CONDUCTIVITY	116.93(7.20)	115.71(1.72)	0.273
SCATTER	59.59(9.30)	58.806(1.99)	1.000

*p value is significant when <0.05

Table 12: Comparison of the CPD parameters for monocyte among MDS and non MDS patients:

MONOCYTES	**MDS cases (n-68)**	**NON MDS cases (n-155)**	**p-value**
	Mean(SD)	Mean(SD)	
VOLUME	176.43(16.06)	165.66(7.621)	0.000
CONDUCTIVITY	124.76(7.904)	125.60(4.22)	0.711
SCATTER	75.82(10.18)	75.83(5.03)	1.000

*p value is significant when <0.05

Table 13: Comparison of the CPD parameters for monocyte among MDS and healthy donors:

MONOCYTES	**MDS cases (n-68)**	**Donors (n-98)**	**p-value**
	Mean(SD)	Mean(SD)	
VOLUME	176.43(16.06)	164.80(5.41)	0.000
CONDUCTIVITY	124.76(7.904)	122.47(2.35)	0.009
SCATTER	75.82(10.18)	70.45(5.88)	0.000

*p value is significant when <0.05

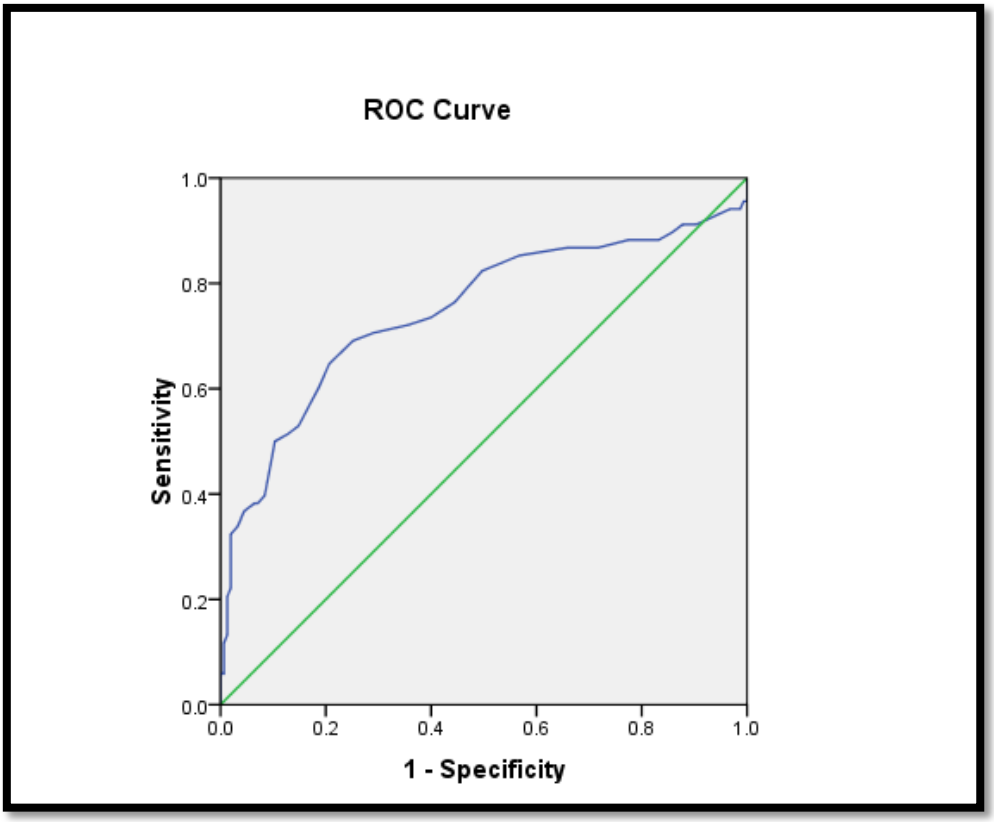

Figure 8: ROC curve for monocyte volume.

By assigning the cut off value for mean monocyte volume as 166.50, the sensitivity was found to be 73.5% and the specificity was 60.0%.

Table 14: Comparison of the CPD parameters for eosinophils among MDS and non MDS patients:

EOSINOPHILS	**MDS cases (n-68)**	**NON MDS cases (n-155)**	**p-value**
	Mean(SD)	Mean(SD)	
VOLUME	154.95(22.61)	156.79(7.81)	0.907
CONDUCTIVITY	152.63(15.83)	150.39(9.28)	0.355
SCATTER	176.87(16.58)	185.50(5.42)	0.000

*p value is significant when <0.05

Table 15: Comparison of the CPD parameters for eosinophilsamong MDS and non MDS patients:

EOSINOPHILS	**MDS cases (n-68)**	**Donors (n-98)**	**p-value**
	Mean(SD)	Mean(SD)	
VOLUME	154.95(22.61)	158(6.04)	1.000
CONDUCTIVITY	152.63(15.83)	148.36(2.87)	0.340
SCATTER	176.87(16.58)	184.22(4.77)	0.799

*p value is significant when <0.05

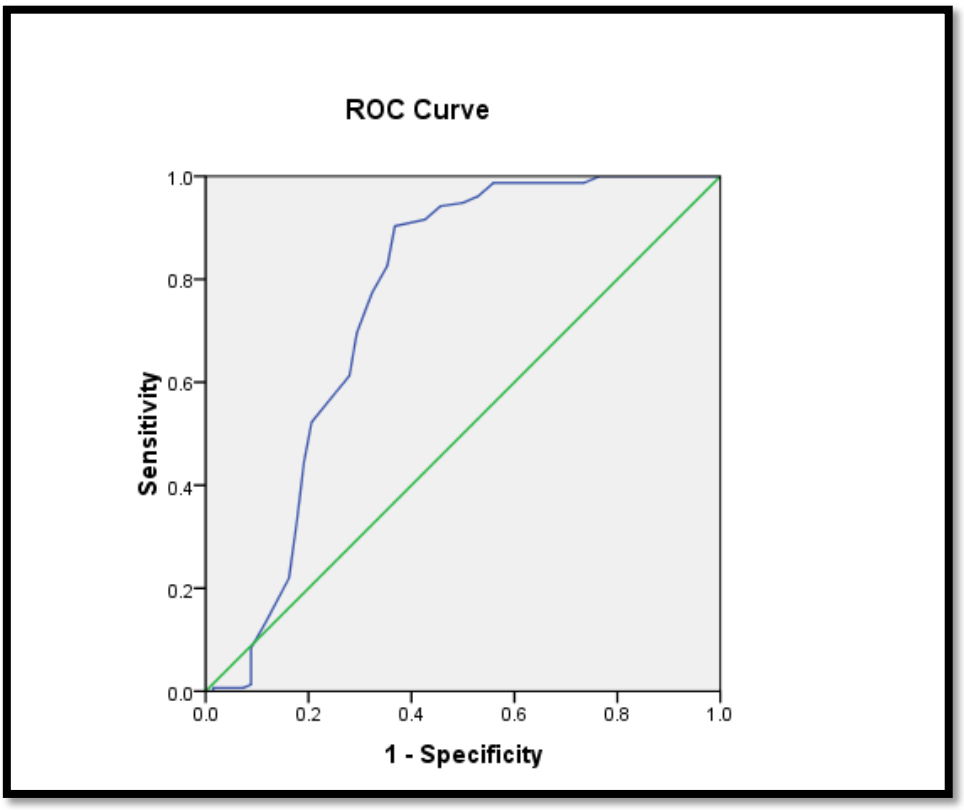

Figure 9: ROC curve for eosinophil scatter.

By assigning the cut off value for mean eosinophil scatter as 180.50, the sensitivity was found to be 90.3% and the specificity was 63.2%.

Table 16: Comparison of CPD data among the low grade and high grade cases of MDS.

CPD PARAMETERS	MDS		p-value
	LOW GRADE	HIGH GRADE	
NEUTROPHIL			
Volume (±SD)	151.32 (15.967)	156.68(16.784)	0.183
Conductivtiy (±SD)	146.65(5.663)	142.45(6.397)	0.006 (p-val <0.05)
Scatter(±SD)	127.68(11.846)	122.42(16.749)	0.135
LYMPHOCYTES			
Volume(±SD)	89.57(6.902)	92.35(10.378)	0.191
Conductivity(±SD)	118.05(3.944)	115.58(9.695)	0.160
Scatter(±SD)	59.84(7.049)	59.29(11.559)	0.811
MONOCYTES			
Volume(±SD)	174.16(14.673)	179.13(17.439)	0.207
Conductivity(±SD)	126.38(5.992)	122.84(9.455)	0.065
Scatter(±SD)	74.86(8.638)	76.97(11.817)	0.401
EOSINOPHILS			
Volume(±SD)	156.08(12.939)	153.61(30.628)	0.678
Conductivity(±SD)	152.19(14.281)	153.16(17.750)	0.803
Scatter(±SD)	176.32(13.544)	177.52(19.832)	0.770

ASSESSMENT OF THE MICROVESSEL DENSITY AND COMPARISON BETWEEN MDS PATIENTS AND CONTROLS:

Out of the total 111 cases included in this study, the paraffin embedded tissue blocks were available for 107 cases. The remaining 4 cases were slides for review that was referred from outside hospital and hence blocks were not available. The MVD was calculated in the remaining 107 cases.

Among the 107 cases, in 6 cases, there was no positive staining of endothelial lined vessels using anti CD34 antibody even after repeat staining. Hence these 6 cases were considered inconclusive for the enumeration of MVD. Consequently, only the results obtained from the 101 cases are discussed throughout the study.

The age distribution ranged from 3 years to 78 years. Among the 101 cases, 68 were males and 33 were females.

Microvessel density(MVD):

Table 17: The mean MVD of MDS cases and controls.

	Micro Vessel Density (per high power field 40X)		p-value
	MEAN	SD	
MDS patients (101)	8.836	5.961	0.000
Controls (35)	5.531	2.502	

The microvessel density (MVD) is significantly different between MDS cases and controls with a t statistic of 4.536 and p-value of 0.000.

Table 18: Mean MVD in controls andamong different WHO subtypes

	Number of subjects	Mean MVD per high power field
Controls	35	5.531
RAEB-I	20	10.36
RAEB-II	17	8.411
MDS-F	2	14.5
RCUD	4	10
RCMD	30	8.866
5q deletion	6	4.733
RARS	5	7.6
Hypoplastic MDS	8	7
MDS-U	7	10.82
Childhood MDS	2	4.5

Cellularity and MVD:

The cellularity of the marrow was assessed subjectively and was classified as hypercellular, normocellular and hypocellular for the respective ages.

Among the 101 cases, 74 cases had hypercellular, 8 cases had normocellular and 19 cases had hypocellular bone marrows respectively.

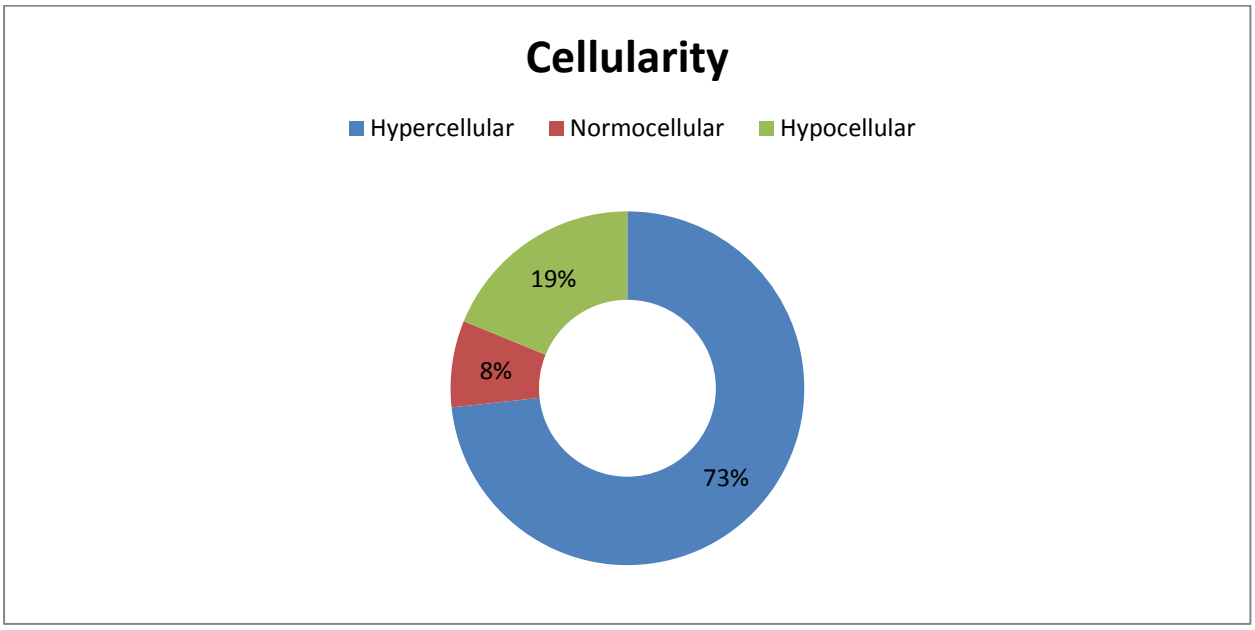

Figure 10: Distribution of cellularity of bone marrow among MDS patients.

The mean MVD was higher in cases with hypercellularity of the marrow.

Table 19: Comparison of bone marrow cellularity and MVD:

Cellularity	MVD (per high power field 40x)	
	Mean	SD
Hypercellular	9.824	6.5565
Normocellular	6.200	3.0817
Hypocellular	6.095	2.0853

Blast percentage and MVD:

Table 20: Comparison of blast percentage and MVD:

MDS cases	**MVD**		**p-value**
	Mean	**SD**	
Low grade	8.277	4.6976	0.287
High grade	9.723	7.5347	

Based on the blast percentage, the cases were classified as low grade and high grade. There were 62 low grade cases and 39 high grade cases. There was no significant difference in the MVD between the low and high grade cases.

The vessels that were observed showed differences in the morphological patterns.

The different morphological patterns include:

1. Vessels that were more or less straight in shape with focal branching and a small visible lumen. These vessels had a uniform linear staining of endothelial cells by CD34 antibody.

2. Large vessels with visible lumina and irregular, branching shapes.

3. Sinusoid like vessels.

4. Small vessels without a visible lumen which appeared to be like "endothelial sprouts"

However, there was a mixture of all these patterns in a given case and did not have any relation to the type or cellularity of the marrow.

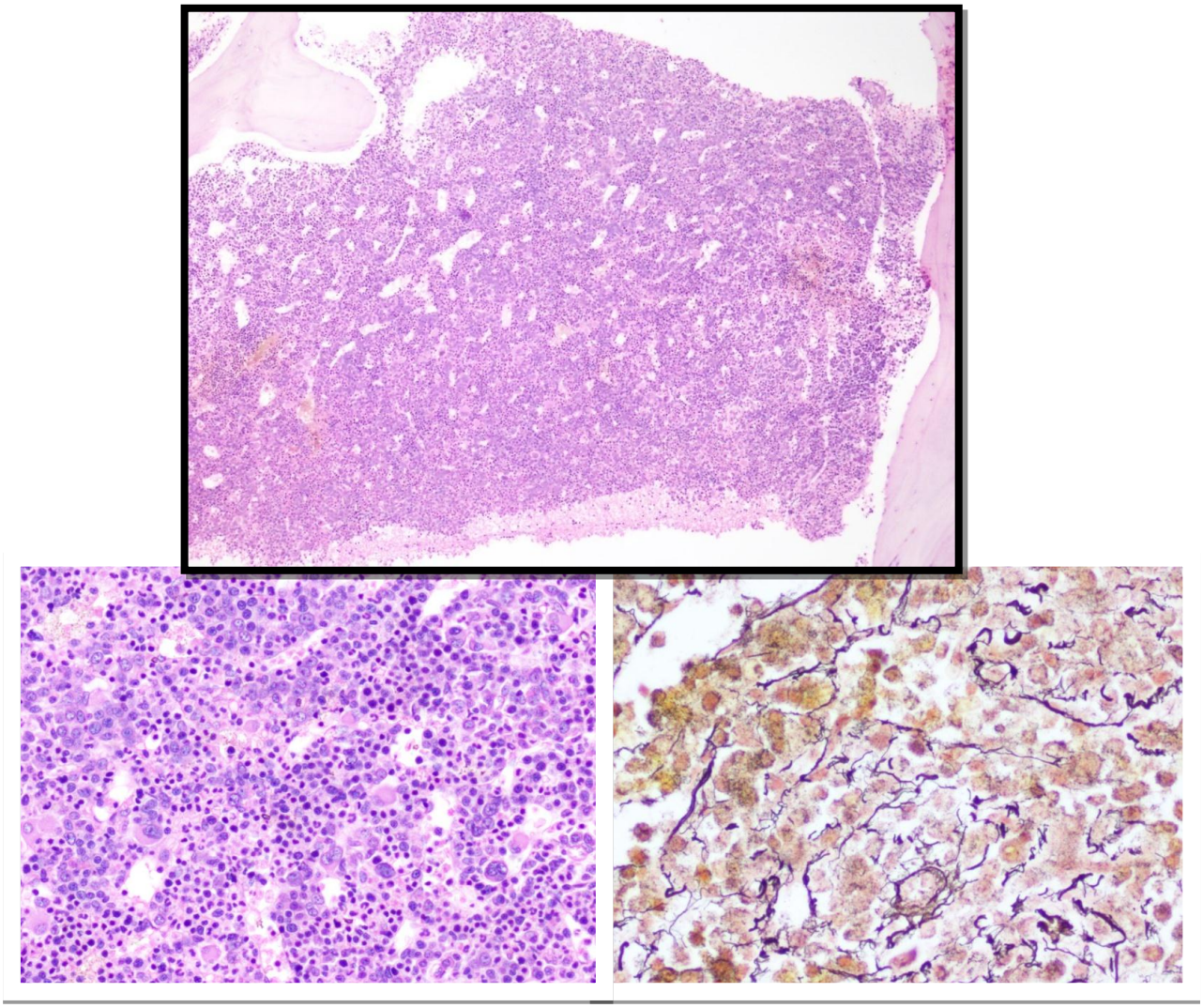

Fig 11: Hypercellular marrow in a case of MDS (a) showing marked erythroid hyperplasia (b) and an increased reticulin content (c).

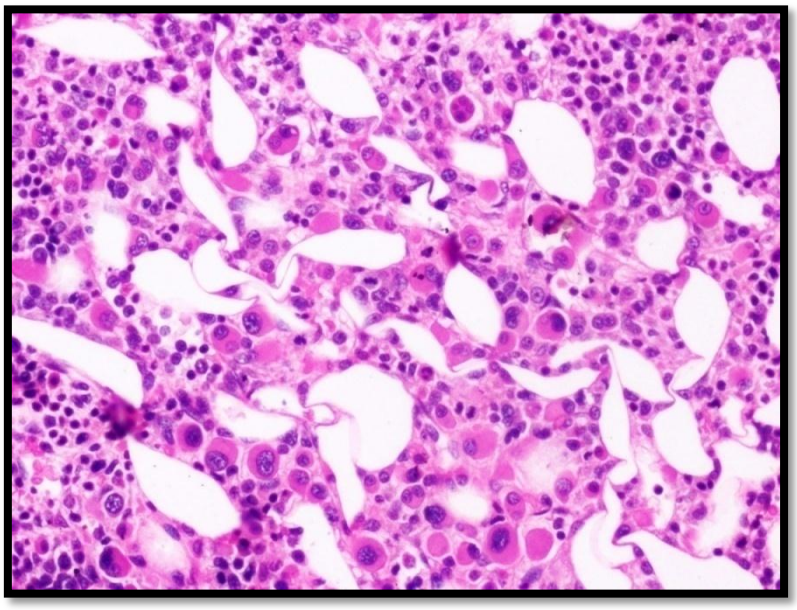

Figure 12: Megakaryocyte hyperplasia and dysmegakaryopoiesis – an example of cellular alterations seen in MDS.(20x, H&E)

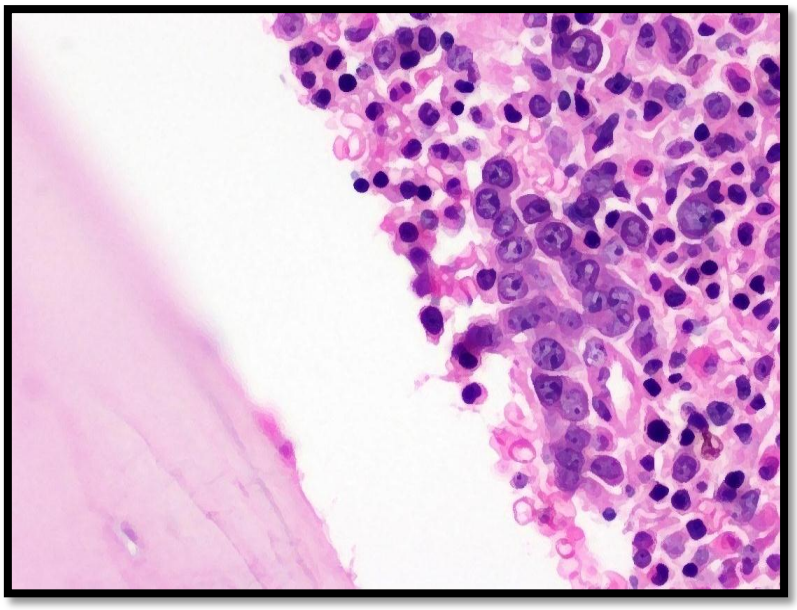

Figure 13: Paratrabecular immature erythroid colonies – an example of stromal alterations seen in MDS.(40x, H&E)

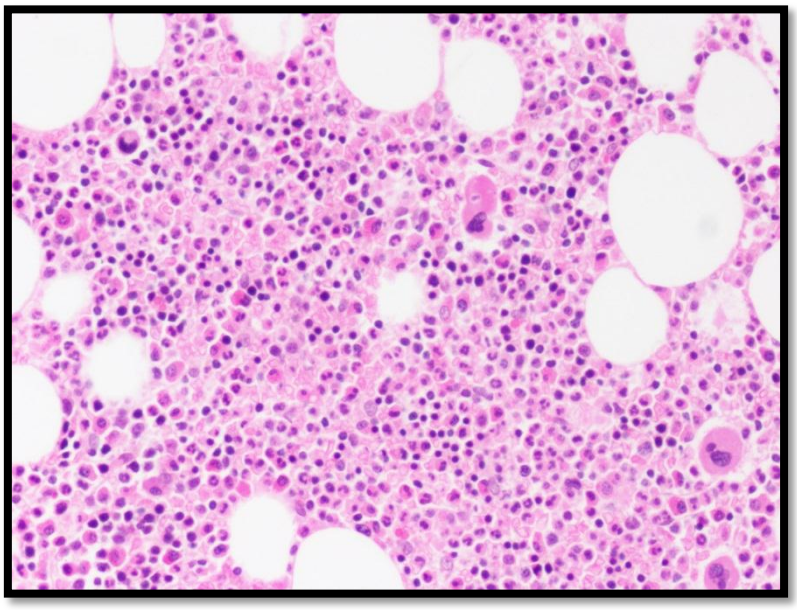

Figure 14: Mildly hypercellular marrow from a 56 year old male patient without MDS . (20x, H&E)

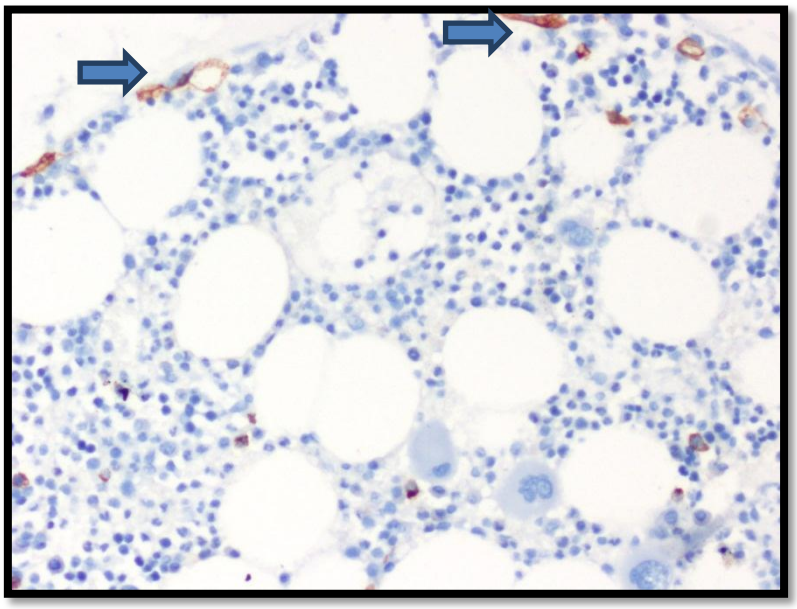

Figure 15: The corresponding IHC (anti CD34) section of the field in fig 14 showing only few thin linear vessels in the paratrabecular location. (arrow heads) (20x)

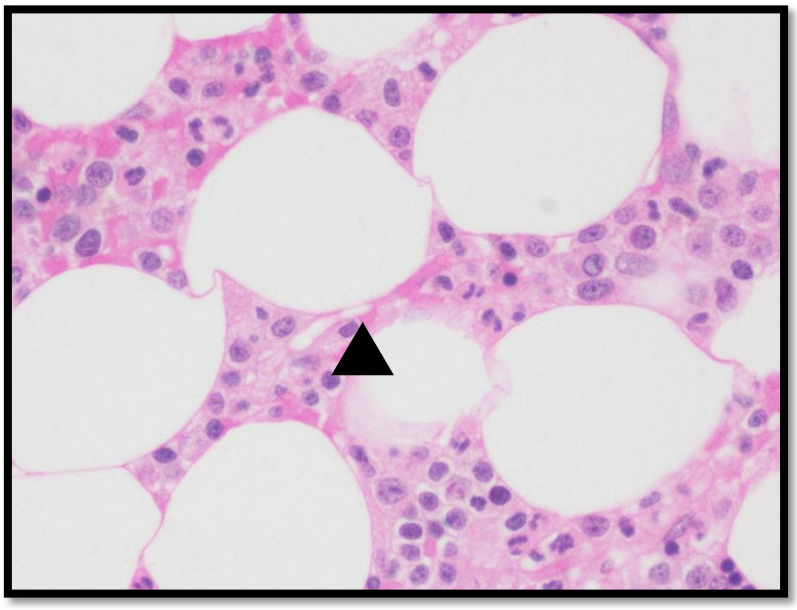

Fig 16: High power view of a linear thin walled patent capillary in a patient without MDS. (40x, H&E)

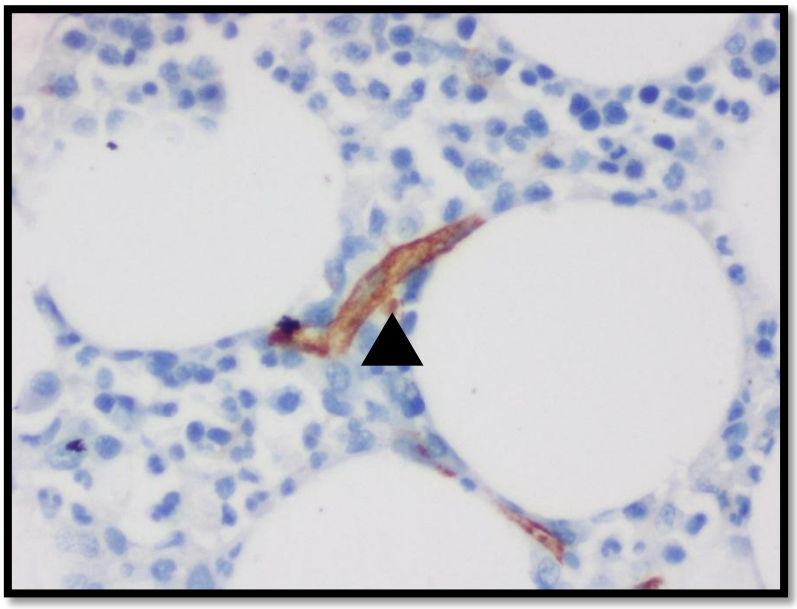

Fig 17: Anti CD34 immunostained sections of the corresponding field in Fig 16 (40x)

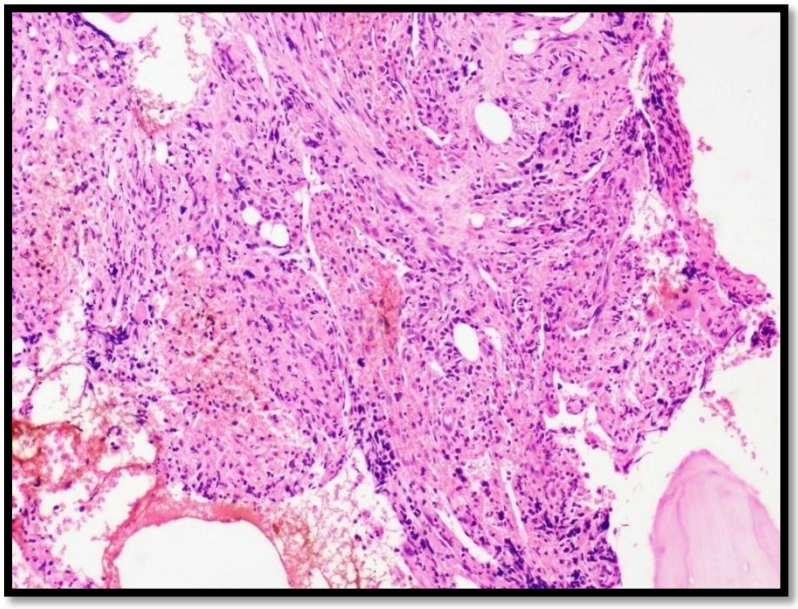

Figure 18: A case of MDS with increase in microvessel density (MVD). (10x, H&E)

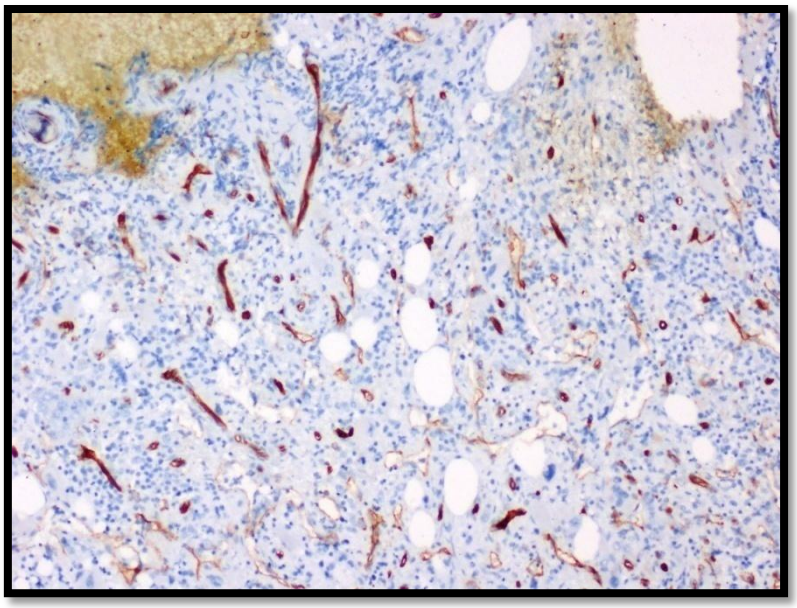

Figure 19: The corresponding field in Figure18 immunostained with anti CD34 antibody demonstrate an increase in the MVD. (10x)

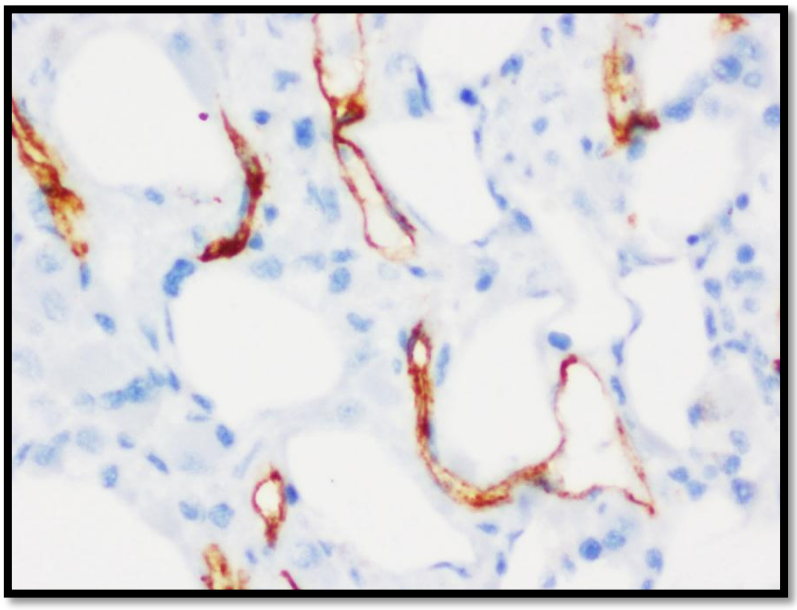

Figure 20: Irregularly dilated endothelium lined vessels. (40x, antiCD34)

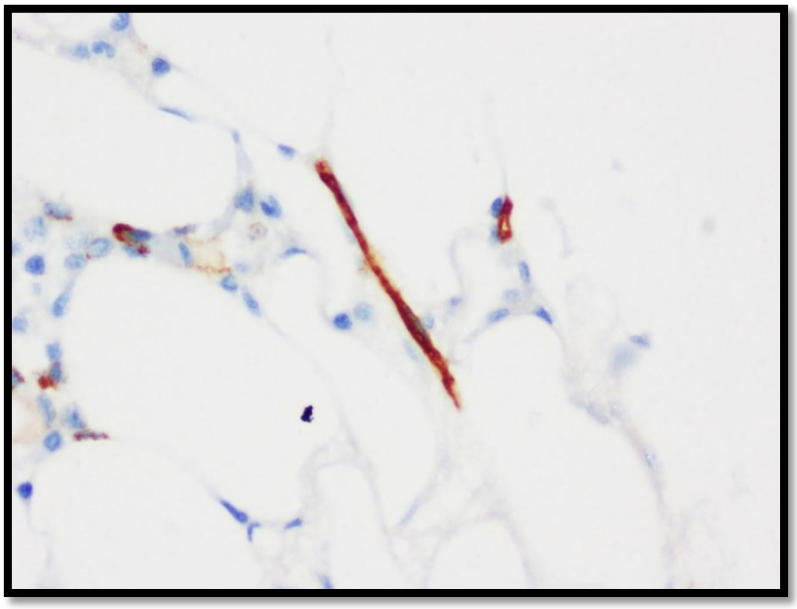

Figure 21: A single linear microvessel which shows immunostaining with anti CD34 antibody. (40x)

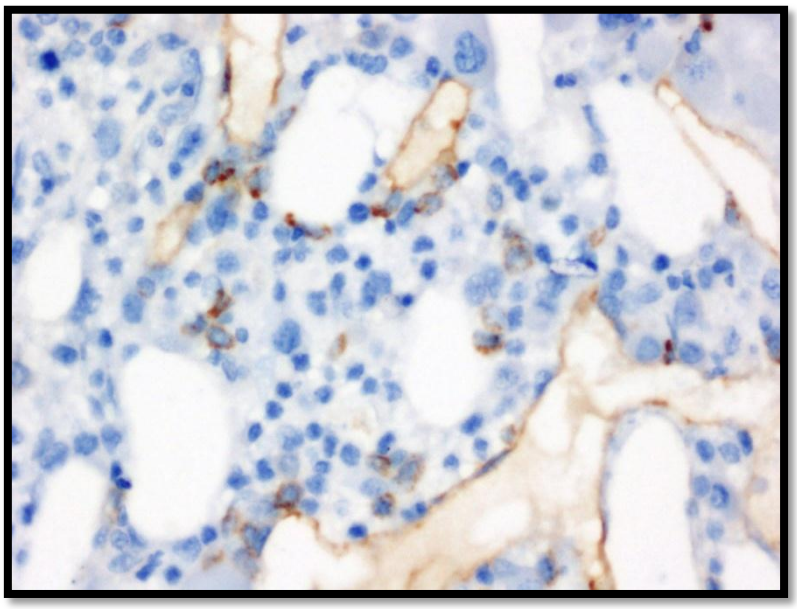

Figure 22: Sinusoid like vessels with dilated, irregular lumen. (40x, anti CD34)

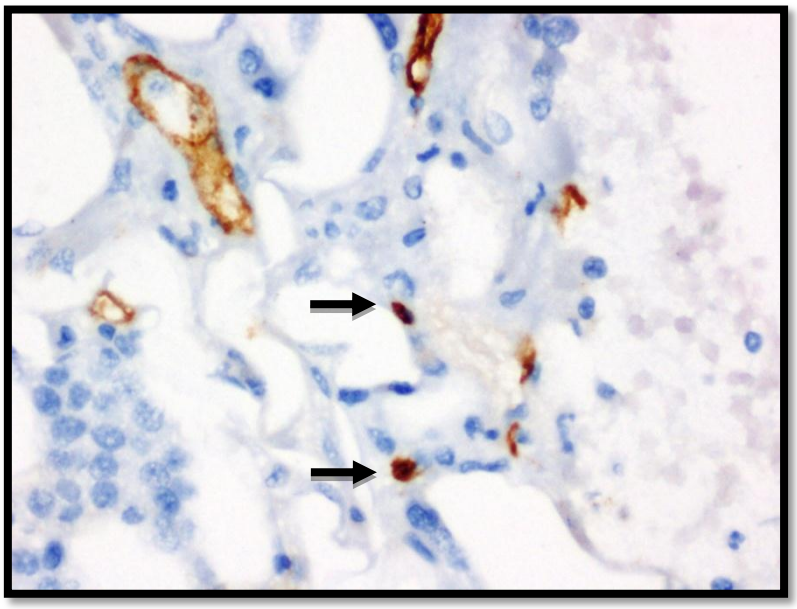

Figure 23: Small endothelial sprouts with no visible lumen and strong immunoreactivity for anti CD34 antibody. (40x)

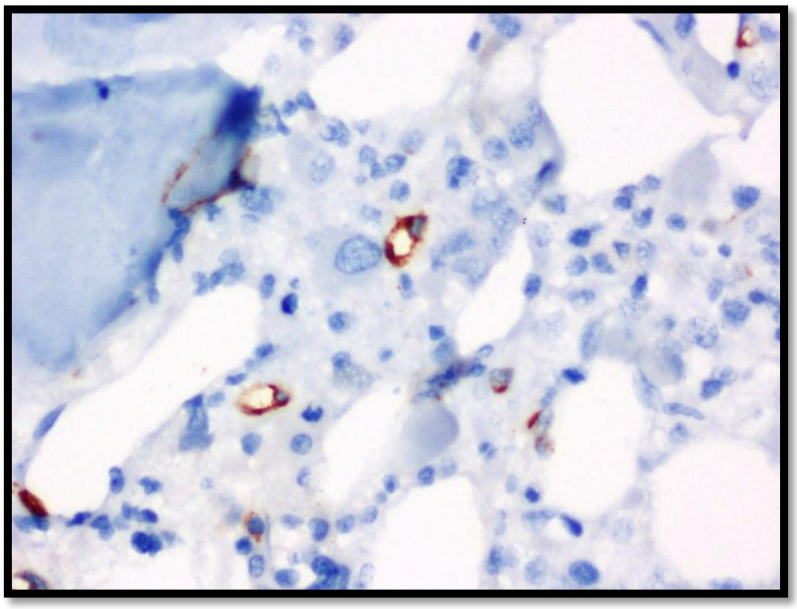

Figure 24: Two microvessels cut in cross section displaying a visible lumen and positively staining endothelial cells for anti CD34 antibody. (40x)

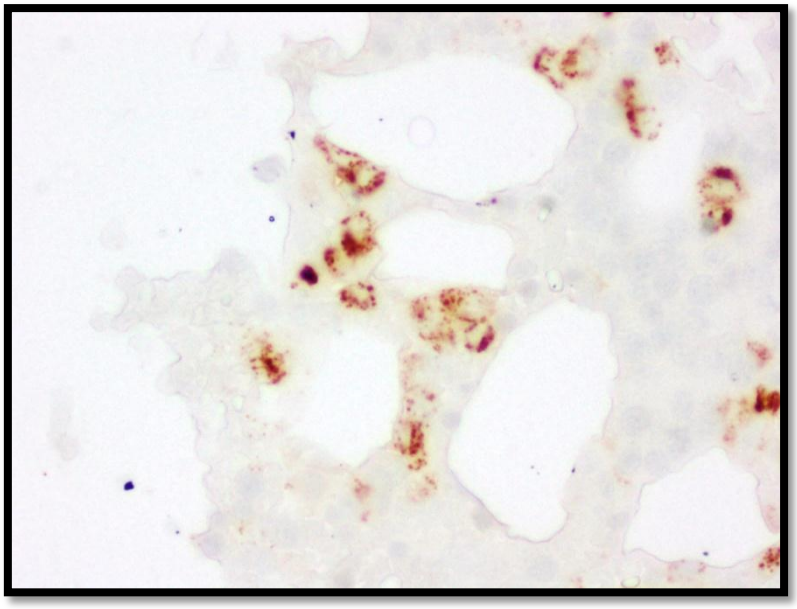

Fig 25: A group of blasts which show a granular staining of the cytoplasm with antiCD34 antibody. (40x)

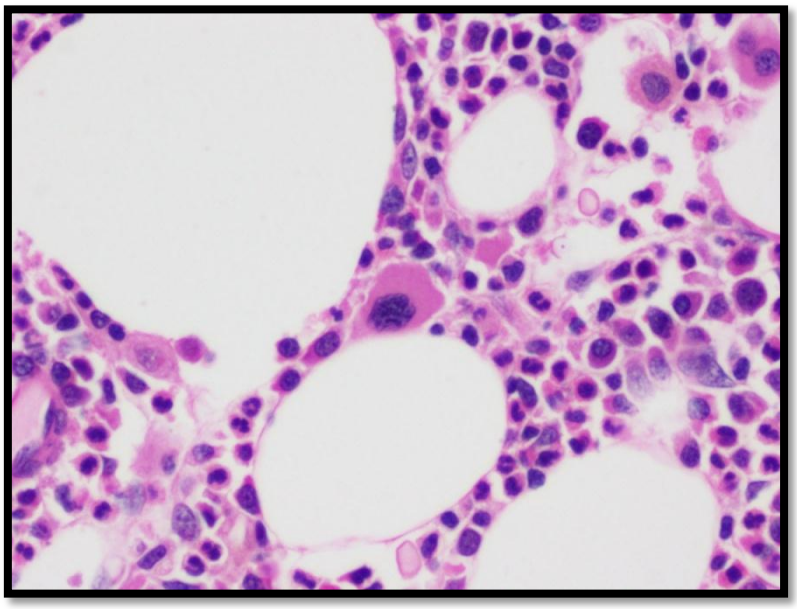

Figure 26: A dysplastic monolobate megakaryocytes in a case of MDS RCMD. (40x, H&E)

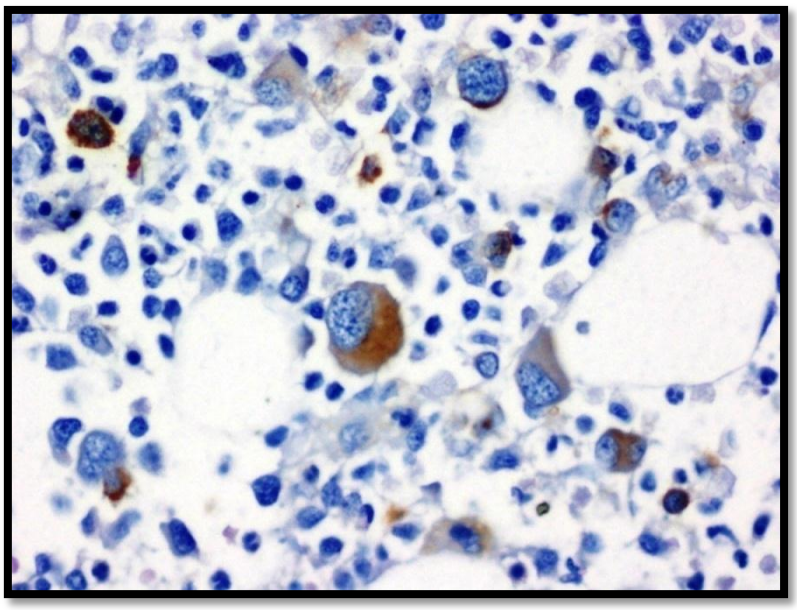

Figure 27: Dysplastic megakaryocytes staining positive with antiCD34 antibody. (40x)

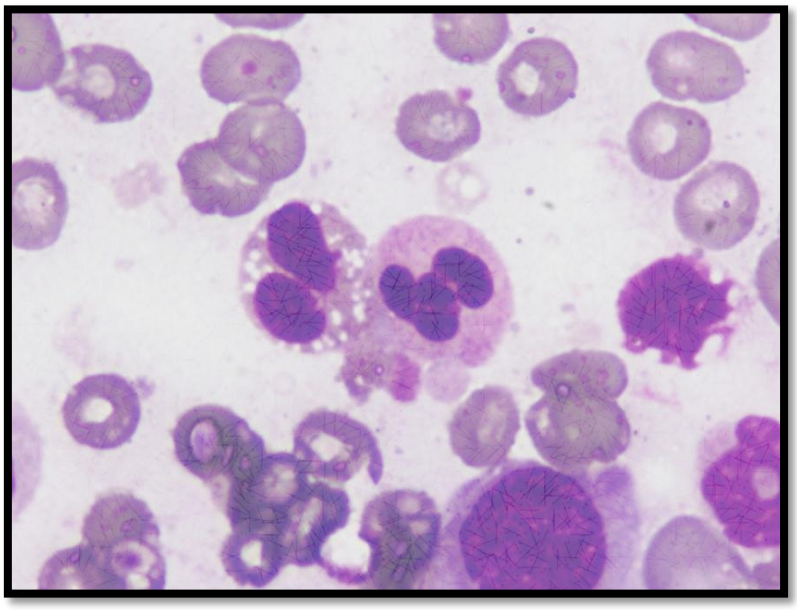

Figure 28: Two developing myeloid cells (Neutrophils) in the marrow with marked pleomorphism in their cellular morphology. (100x, MGG)

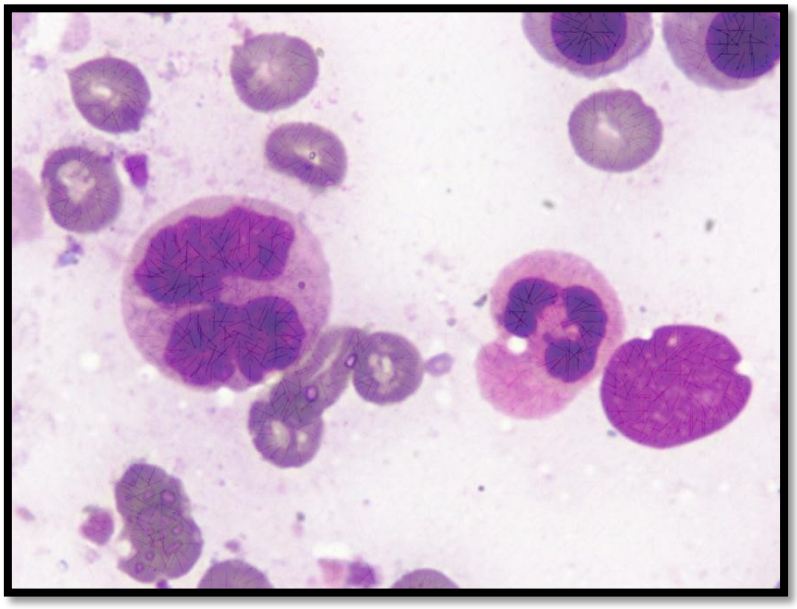

Figure 29: Two developing myeloid cells (Neutrophils) in the marrow. One of the neutrophils (left side) shows features of dysplasia. (100x, MGG)

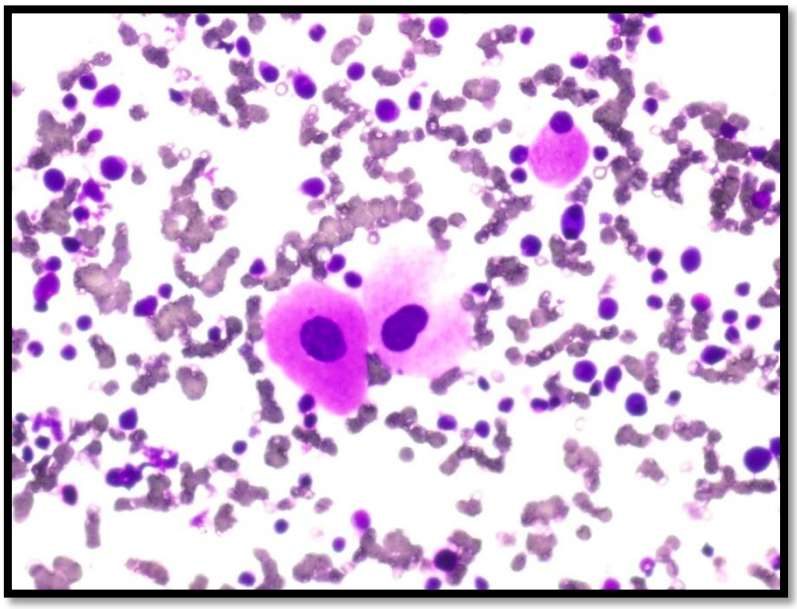

Figure 30: Three monolobate megakaryocytes in the bone marrow aspirate of a case with 5q deletion syndrome. (40x, MGG)

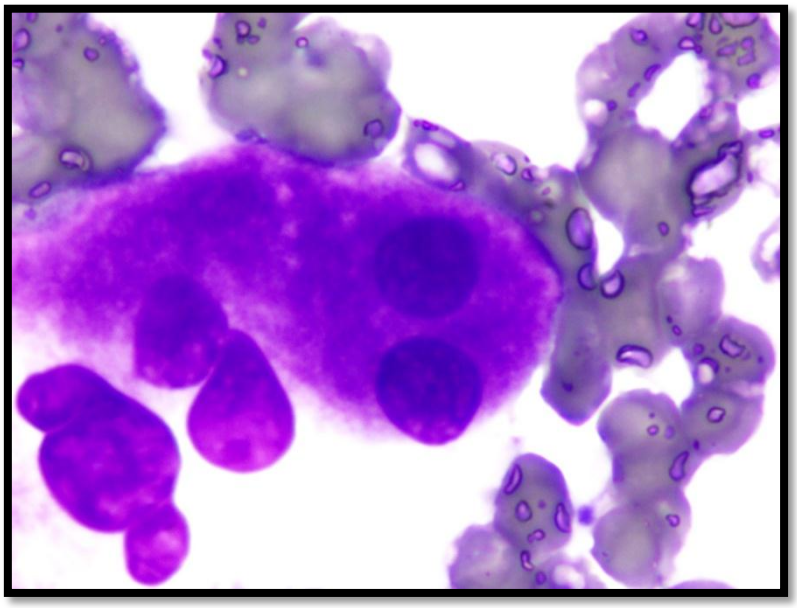

Figure 31: Dysplastic megakaryocyte with two separate nuclear lobes in a case of MDS. (100x, MGG)

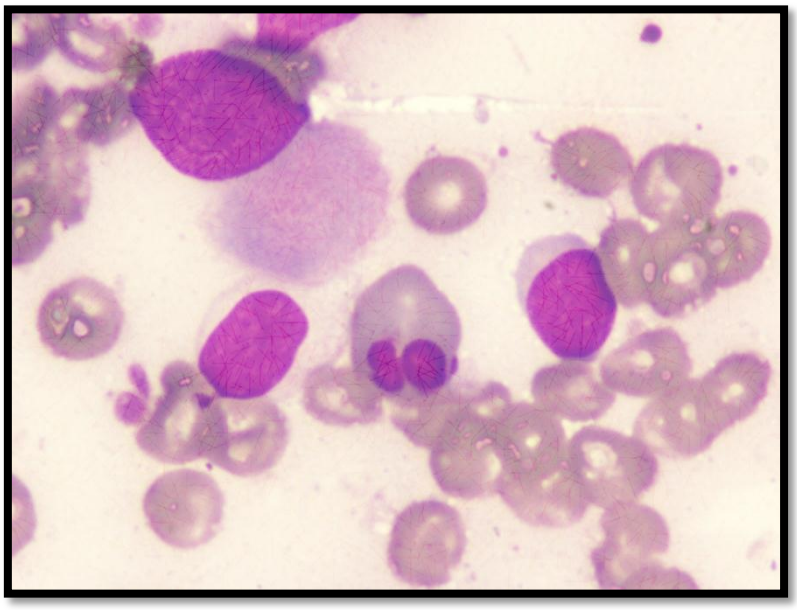

Figure 32: Erythroid dysplasia identified by the presence of nuclear budding. (100x, MGG)

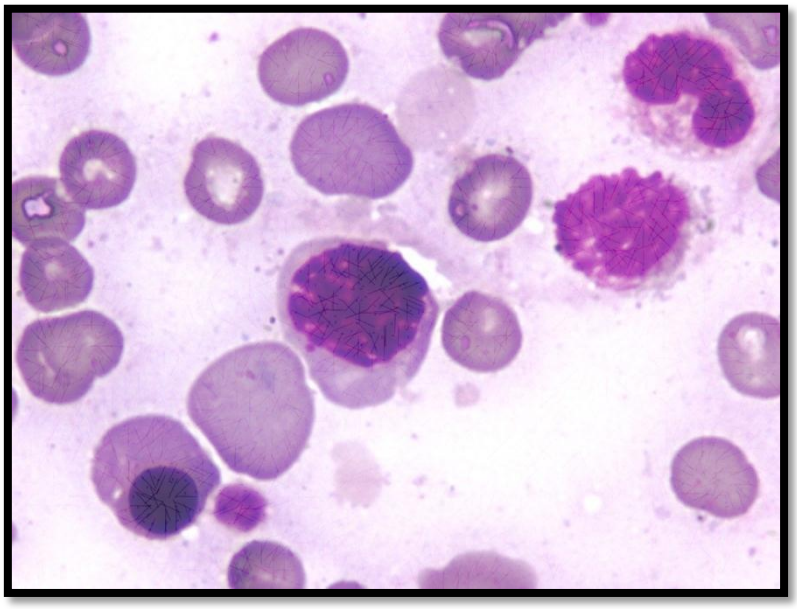

Figure 33: Megaloblastoid erythroid maturation in a case of MDS. (100x, MGG)

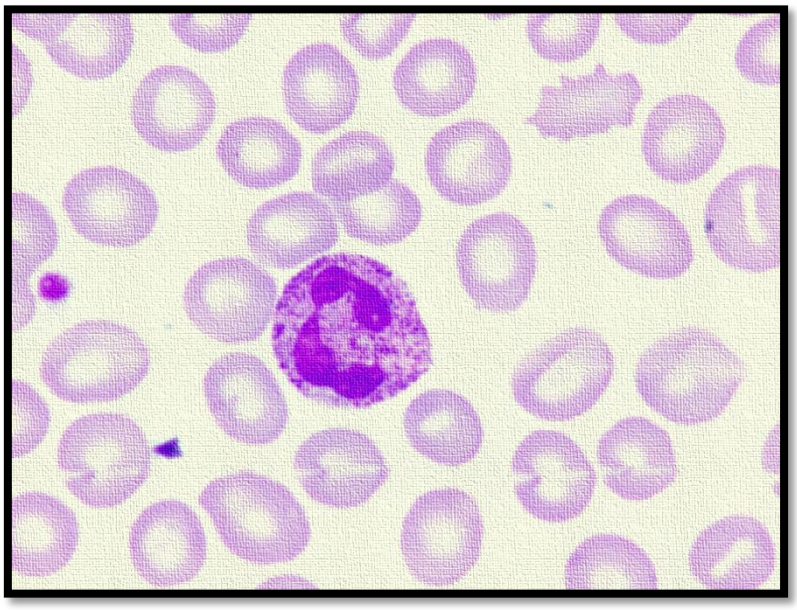

Figure 34: A neutrophil in the peripheral smear of a patient with bacterial infection.The cytoplasm contains toxic granules. (100x, MGG)

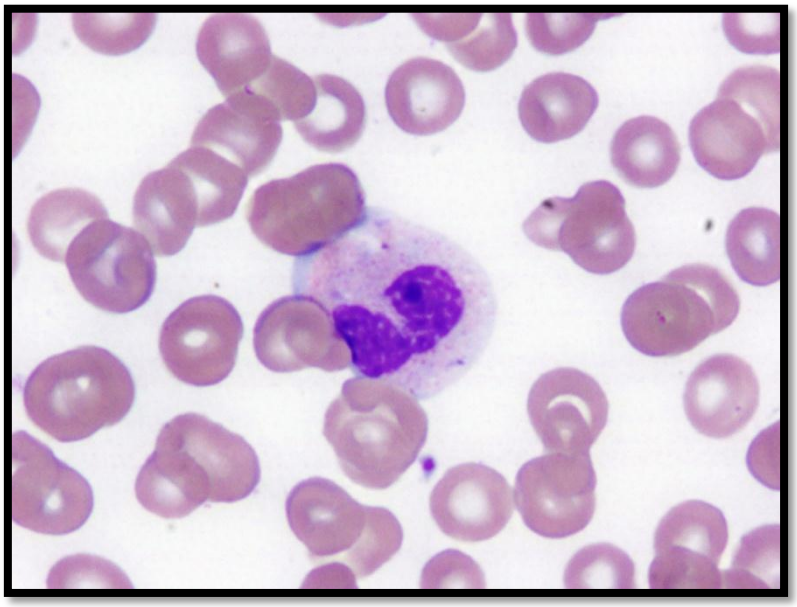

Figure 35: Hypogranular neutrophil in a patient with MDS. The neutrophil also shows the classic pseudo Pelger Huet anomaly seen in cases with MDS. (100x, MGG)

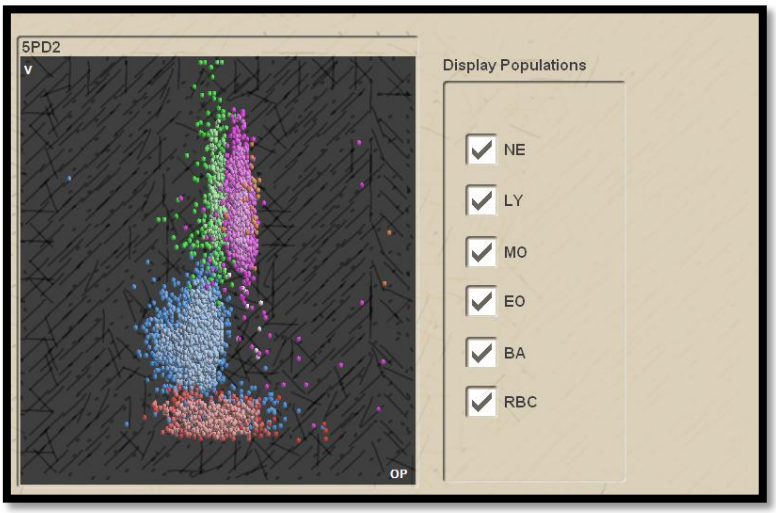

Figure 36: The scatter plot of the 5 part differential WBC count in a case of MDS RCMD. The neutrophil population (pink dots) overlaps with the monocyte population (green dots) due to an increase in the size and decrease in granularity.

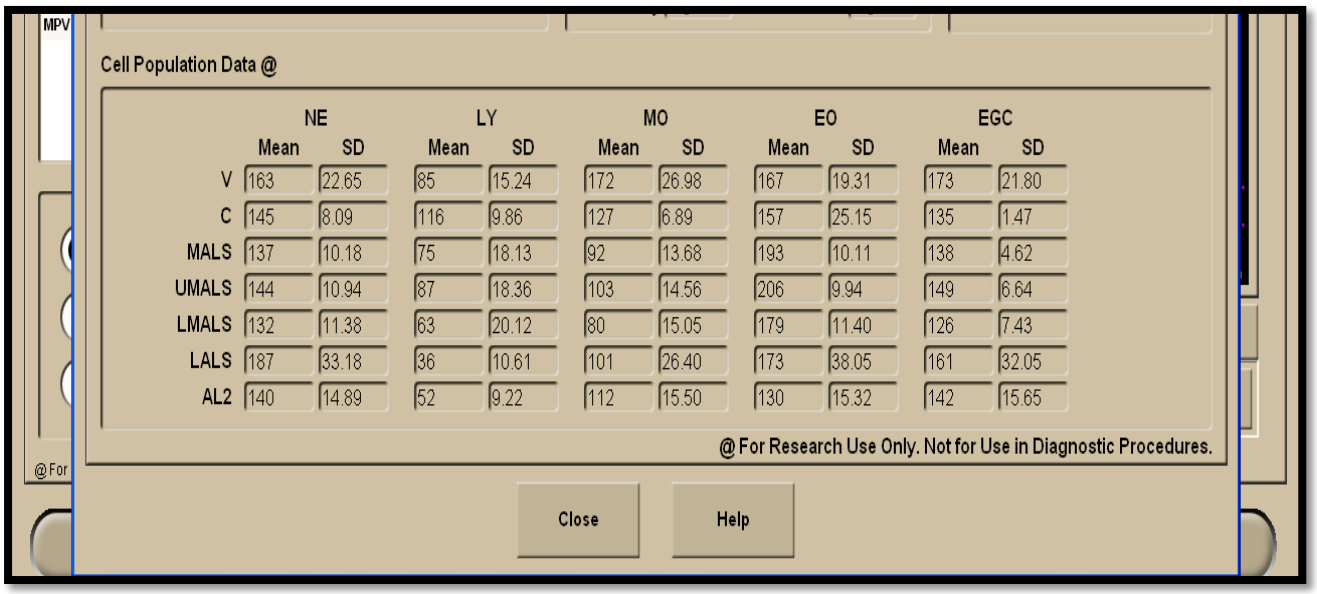

Figure 37: The cell population data of the same case of MDS RCMDfor which the scatter plots are shown in Figure 36.

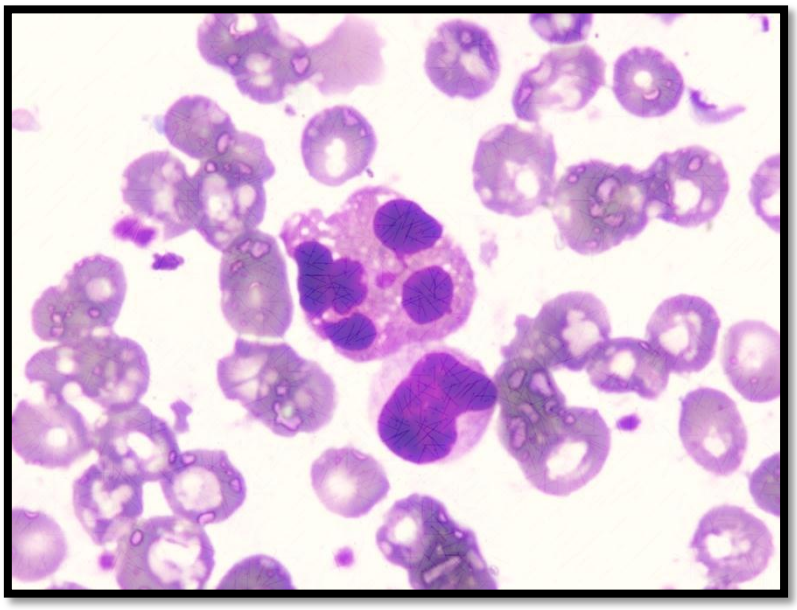

Figure 38: A dysplastic eosinophil in the bone marrow displaying nuclear hyperlobation. (100x, MGG)

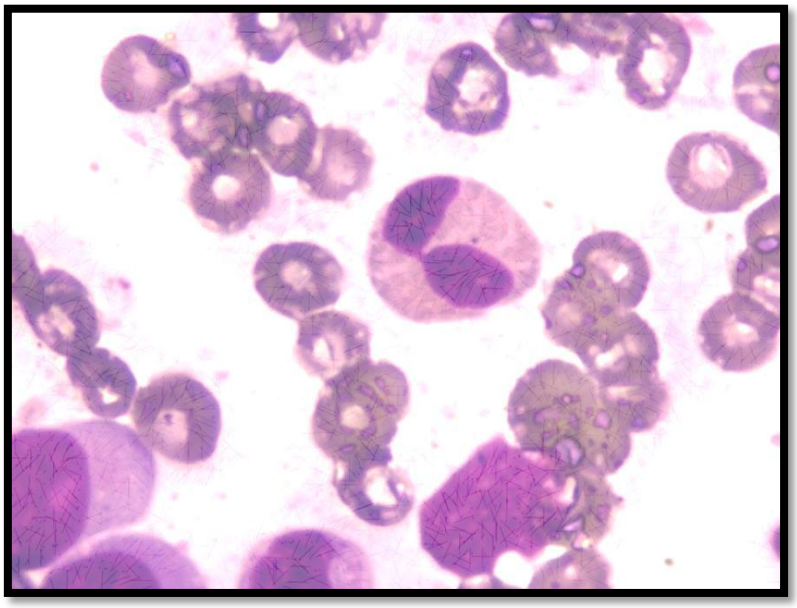

Figure 39: An eosinophil in the bone marrow with hypogranularity in a case of MDS. (100x, MGG)

DISCUSSION

DISCUSSION:

CELL POPULATION DATA:

Myelodysplastic syndrome (MDS) is a clonal disorder, which affects the pluripotent hematopoietic stem cells. Hence the abnormal development is reflected in all the major cells lineages namely the erythroid, myeloid and the megakaryocytic lineages. Although there are well-documented features of dysplasia in the red blood cells and the platelets, the dysplastic changes are easily recognised and appreciated in the cells belonging to the myeloid series. This is partly because of the fact that the traditional staining procedure employed for staining of the peripheral blood film, using Romanowsky stains, imparts a differential staining to the white blood cells so that distinct identification of granulocytes such as neutrophils, eosinophils, basophils and agranulocytes such as monocytes and lymphocytes can be made based on the cytoplasmic staining, colour of granules and the nuclear features. In addition, the morphology of erythrocytes can be influenced by co-existing common conditions such as iron deficiency anaemia. The morphological changes observed in the peripheral blood smear arealso reliably reflected by automated cell counters, contributing to the generation of "suspect flags".

The five part differential counts of the white blood cells on the Beckman Coulter DXH 800automated haematologyanalyser is determined by the Volume, Conductivity and Scatter principle. The values measured by the cell counter in terms of size, conductivity and scatter corresponds to the light microscopic findings such as macrocytosis, differences in the granularity, nature of the granules and nuclear complexity respectively. This can be termed as "Machine morphology".

Apart from the traditional numerical counts of the blood cells (Complete Blood Counts or CBC), histograms and scatter plots are also provided by the automated cell counters. The patterns in the histograms and scatter plots can also be utilised to understand the distribution, size and property of the cells. The following part of the discussion enumerates the significant changes observed in this study and describes parameters that may assist in predicting patient samples with MDS.

In the present study, the cell population data was studied for the four major classes of leukocytes namely neutrophils, lymphocytes, monocytes and eosinophils. In human blood, neutrophils contribute to the major population of the white blood cells. Hence the search for an abnormality of the neutrophil series would be more rewarding and can be easily seen.The study showed a significant difference between the volume, conductivity and the scatter of the neutrophils between the MDS and the non-MDS cases.

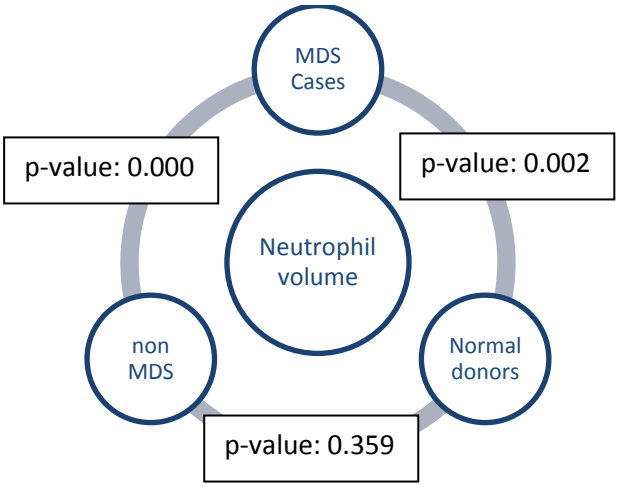

Figure 40: Difference in the mean neutrophil volume (MNV) between theMDS, non MDS and donor groups.

COMPARISON OF CPD PARAMETERS OF NEUTROPHILS

The mean neutrophil volume (MNV) in non MDS cases and the healthy blood donors were 147.08 and 148.90 respectively. On the contrary, in MDS cases, there was an increase in the mean volume of neutrophils up to 153.76. This difference in volume of the neutrophils between cases of MDS in comparison to those of non MDS and the normal controls was statistically significant. (Fig : 40)

There is ample evidence that the mean neutrophil volume is also higher is cases of megaloblastic anaemia(s) (MA) when compared to normal. This increase in the volume has been attributed to the fact that the maturationis characterised by nuclear-cytoplasmic asynchrony occurring in the marrow of patients with MA.The increase in the cell volume can also be attributed to the fact that there is nuclear

hypersegmentation in neutrophils. Patients with sepsis who manifest toxic changes in the neutrophils also have a high neutrophil volume.

This increase in the mean neutrophil volume is possibly because of the presence of immature neutrophil precursors in the peripheral blood. When there is a left shifted neutrophil maturation in the peripheral blood, the cell counter not only measures the mature neutrophils, but also the immature forms such as myelocytes, metamyelocytes which have a higher volume. This mechanism can be accounted for the increase in the mean neutrophil volume of patients with sepsis. (44)

Patients with MDS also manifest a megaloblastoid maturation of the hematopoietic cells which is commonly seen in the erythroid series of cells and is reflected in the routine peripheral blood film as macrocytosis. Since the disorder is clonal,the neutrophils and other myeloid cells also undergo a megaloblastoid maturation similar to that seen in red blood cell precursors. Immature neutrophil precursors such as myelocytes and metamyelocytes are also present in the peripheral blood of patients with MDS. Hence both these mechanisms might help us explain the increase in the mean neutrophil volume noted in the study.

In cases of MDS, the neutrophils frequently exhibit features of cytoplasmic vacuolations similar to those in sepsis and in patients with reactive neutrophilia. The cytoplasmic vacuolations can also be considered a possible cause for the increase in the mean volume of neutrophils.

Another possibility to entertain is, whether an increase in the MNV can be attributed to the mechanism of apoptosis. This may be significant since peripheral cytopenia noticed in cases of MDS is ascribed to enhanced apoptosis in precursor cells. As per definition, apoptosis is classically associated with shrinkage in the volume of cell. (30)On the contrary, there is an increase in the MNV observed in this study. At this point, there is a need to highlight a newly discussed term ”Necroptosis”.

Necroptosis is a form of cell death that involves a mixture of processes seen in necrosis and apoptosis. Biochemically and morphologically, the process resembles necrosis. There is loss of ATP, cell

swelling, release of lysosomal enzymes and rupture of the plasma membrane. But, unlike necrosis the stimulus for the cell death is triggered by genetically programmed signal pathways. In this manner, the process resembles apoptosis. Because of the combination of both these features, necroptosis is sometimes called "programmed necrosis".(30)

This concept of necroptosis can also explain the changes in neutrophils found in this study. The increase in the neutrophil volume can be attributed to the cellular swelling before the programmed cell death is genetically initiated. The peripheral cytopenia can also be explained likewise.

MEAN NEUTROPHIL CONDUCTIVITY (MNC):

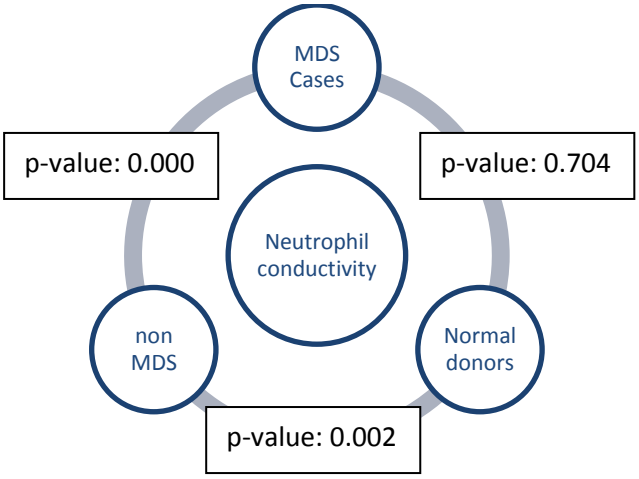

Figure 41: Difference in the neutrophil conductivity between the MDS, non MDS and the donor groups.

Analysis of the data from the mean channel of neutrophil conductivity showed a significant decrease in the mean conductivity in MDS cases compared to the non MDS group (p-value=0.000). But there was no significant difference in mean conductivity when compared with the normal donors. Also noteworthy was the fact that there was significant difference in the conductivity between the non MDS and the donor groups. From the values that have been

obtained, only the non MDS patients had a significant difference in the mean conductivity with both the groups.

The non MDS group includes all patients with illnesses other than MDS. The illnesses include viral fever to solid tumours. Hence there is a high probability that the neutrophils of these group of patients might show evidence of toxic changes and a left shift in the maturation. Toxic changes in the neutrophils is characterised by increased number and density of the granules. This causes an increase in the conductivity of the neutrophils. Because of the hypogranularity in the neutrophils of MDS, there is a measurable decrease in conductivity.

MEAN NEUTROPHIL SCATTER (MNS):

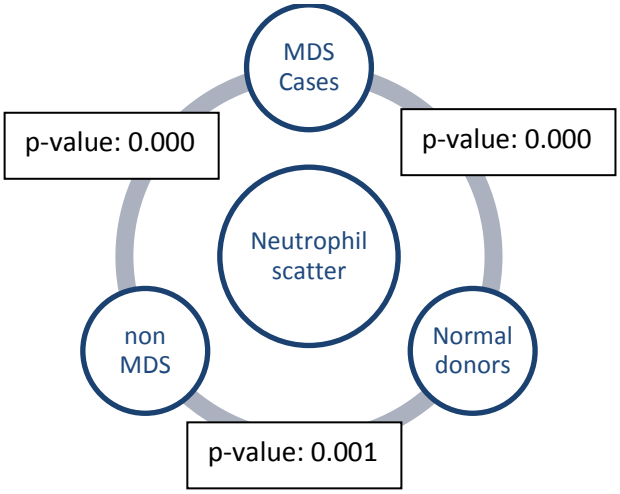

Figure 42: Difference in the mean neutrophil scatter between the MDS, non MDS and donor groups.

In the case of neutrophil scatter, there were significant differences amongst all the three groups. The difference in the content of granularity and the nuclear complexity are two important features reflected by the scatter values. The mean neutrophil scatter values in MDS patients, non MDS patients and healthy donors were was 125.28, 136.94 and 132.88

respectively. These values also reflect the pathology of MDS. Hypogranularity seen in the neutrophils of MDS cases isevidenced as a decrease in the mean neutrophil scatter values.

COMPARISON OF CPD PARAMETERS OF LYMPHOCYTES

The morphology of lymphocytes is not usually considered in the assessment of dysplasia in cases of MDS by light microscopy in the routine setting. Since the lymphocyte is agranular, the point of assessing any difference in the content of granularity does not hold goodwhen assessing for dysplasia. When compared to the other leukocytes, the size of lymphocytes is smaller with a relatively higher nuclear-cytoplasmic ratio. The nucleus of a lymphocyte shows little variation in size and shape when compared to neutrophils and eosinophils.

This study showed a significant difference in the volume of lymphocytes of MDS cases when compared to the non MDS groups and healthy donors (p-value: 0.000 and 0.000 respectively). The mean lymphocyte volume in cases of MDS was increased at 90.84. This value is higher when compared to the non MDS groups and healthy donors who had mean lymphocyte volume of 86.24 and 84.57 respectively.

The reason for an increase in the volume of lymphocytes is debatable. It is possible that there is a minimal increase in the volume of lymphocytes in MDS. This increase in the volume might not be sufficient enough to be perceived at the light microscopic examination of the peripheral smear or the bone marrow.

MEAN LYMPHOCYTE VOLUME (MLV):

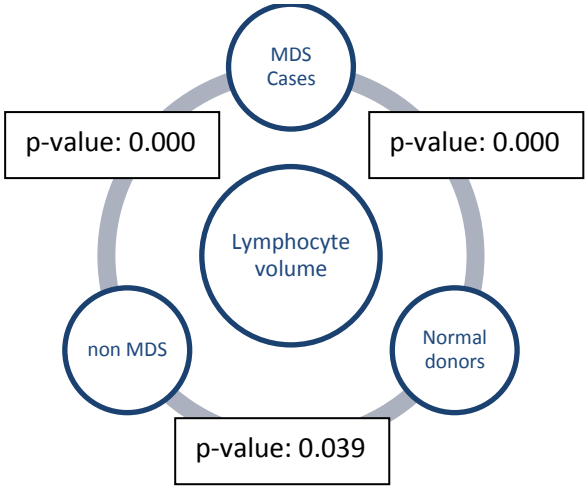

Figure 43: Difference in the mean lymphocyte volume (MLV) between the MDS, non MDS and donor groups.

The figure shows that there is also a significant difference in lymphocyte volume between the non MDS and the healthy donors. This small difference in the volume can be explained by the presence of reactive lymphocytes in the non MDS cases. Reactive lymphocytes morphologically manifest an increase in the volume of cytoplasm as compared to normal.

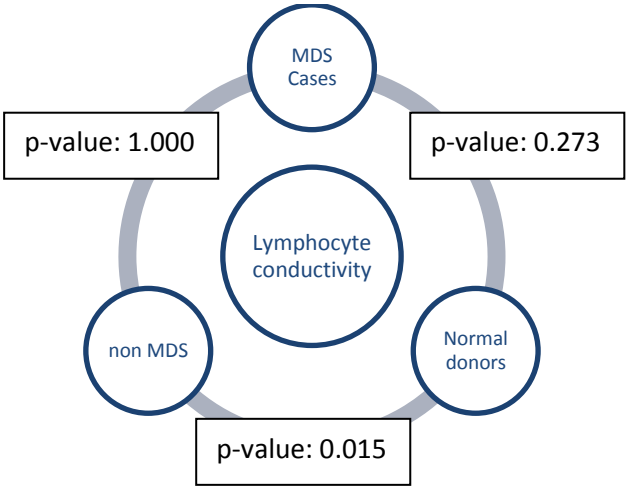

Figure 44: Difference in the mean lymphocyte conductivity (MLC) between the MDS, non MDS and donor groups.

There were no significant differences in the mean lymphocyte conductivity (MLC) and the lymphocyte scatter (MLS) among the various groups of patients and controls. This is acceptable, as lymphocytes are agranulocytes. Hence, only the volume of lymphocytes appeared to be an important parameter to identify MDS.

COMPARISON OF VCS PARAMETERS OF MONOCYTES

Similar to the lymphocytes, monocytes are also agranulocytes. Even though certain authors claim that monocytes can have a fewer number of fine granules, the amount of granules in the cytoplasm of monocytes in negligible when compared to neutrophils and eosinophils. The most striking feature when assessing a monocyte is its size. The monocyte is the largest of the leukocytes in the peripheral blood. Hence changes related to the size of the monocytes may be easier to detectthan the changes in its cytoplasm and the nucleus.

There are several causes of monocytosis in the peripheral blood which include infections, inflammatory conditions and primary hematopoietic neoplasms such as Chronic Myeloid Leukaemia, Acute monocytic leukaemia, myeloproliferative neoplasms and myelodysplastic syndromes. In monocytosis occurring as a part of the primary hematopoietic neoplasms, the monocytes are part of the neoplastic clone.

In acute myelomonocytic leukaemia and acute monocytic leukaemias, the monocytes demonstrate marked immaturity whereas, more mature monocytosis is seen in cases of CML. Monocyte differentiation in the marrow passes through three stages, which include monoblasts, promonocytes and mature monocytes, although neither monoblasts nor promonocytes are identified in the normal marrow specimens.

Though monocytosis has been documented in MDS, the presence of circulating immature forms has not been completely studied. Unlike the neutrophils and eosinophils, definite signs of dysplasia in monocytes are not very clear. In this study, MDS cases recorded a mean monocyte volume (MMV) of about 176.43. This value was significantly high when compared to the non MDS group and the healthy donors with a p-value of 0.000 and 0.000 respectively.

Mardi *et al* and Lee *et al* have demonstrated that there is a significant increase in the mean neutrophil volume and the mean monocyte volume in cases of sepsis. (49)(44)In this particular study, the authors have not discussed about the possible causes for their findings in cases of sepsis. Similar to the neutrophils, the monocytes also show vacuolations and few granules when activated. Thus, alike the neutrophilic series of cells, it is possible that the monocytes also show few immature precursors in the peripheral blood, the differences of which may not be identified at the light microscopy level. The presence of cytoplasmic vacuolations also may contribute to an increase in the volume.

The other two CPD parameters of monocytes such as the conductivity and scatter were not significantly different when compared to the non MDS and the control groups. Thus, the volume was the only parameter that was significantly different among the three groups.

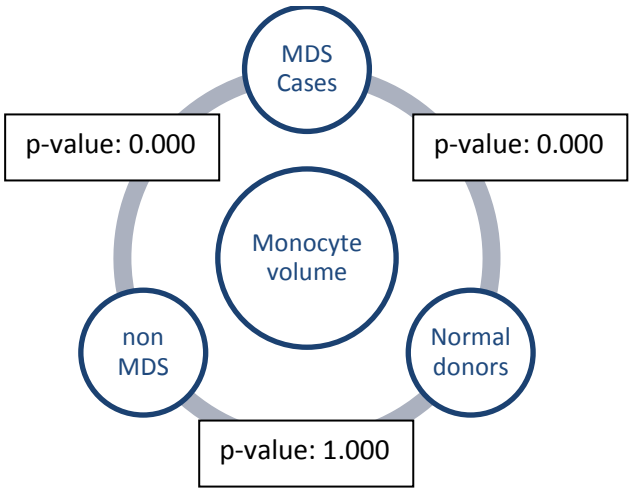

Figure 45: Difference in the mean monocyte volume (MMV) between MDS cases, non MDS patients and donors.

COMPARISON OF CPD PARAMETERS OF EOSINOPHILS

Eosinophils are easily identified in the peripheral smear due to the fact that they have granules in the cytoplasm staining red orange by the conventionally used Romanowskny stains. In the normal healthy individual, they are similar in size to the neutrophils. In this study there was no statistically significant difference in the volume of the eosinophils between the MDS cases and the other groups. The mean eosinophil volume in cases of MDS was 152.96 and that of the non MDS groups and the donor group were 156.79 and 158 respectively. However, the mean volume of the eosinophils (MEV) was considerably lower in comparison with the non MDS groups and the healthy donor group.

The values for conductivity in the eosinophils were also not significantly different amongst the groups. The values for eosinophil conductivity showed a minimal increase compared to the other two groups.

The only significantly different parameter, pertaining to the eosinophils, amongst the three groups was the mean scatter values (MES). The mean eosinophil scatter (MES) was decreased in cases with MDS compared to the non MDS and the donor group.

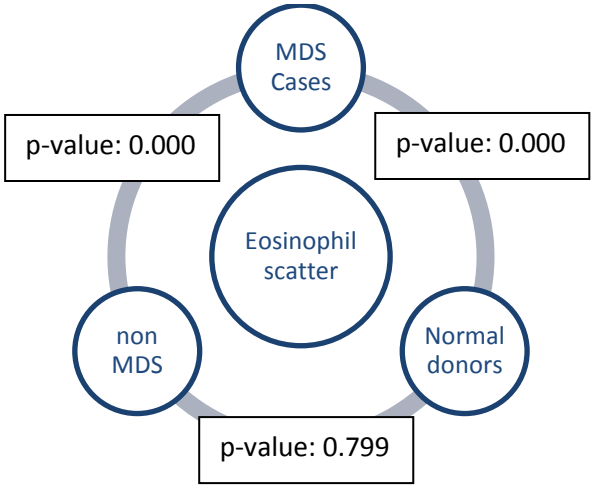

Figure 46: Difference in the mean eosinophil scatter (MES) between the MDS, non MDS and donor groups.

As mentioned in the earlier discussions, the scatter values are greatly influenced by the granularity seen the cytoplasm of the cells. Similar to the neutrophils, eosinophils also show a decrease in the scatter values. The reason for the decrease in the scatter values can be attributed again to the hypogranularity seen in the eosinophils. It is known that a decrease in the granular content of the leukocytes is a feature of dysplasia in cases with MDS. Hence, similar to the neutrophils, the decrease in the granularity in the cytoplasm of the eosinophils can possibly explain the decrease in the mean eosinophil scatter values.

In summary, the CPD parameters of neutrophils, lymphocytes, monocytes and eosinophils were compared between MDS cases, non MDS cases and healthy donors. There was a significant difference in the neutrophil volume (MNV), neutrophil conductivity (MNC),

neutrophil scatter(MNS), lymphocyte volume (MLV), monocyte volume (MMV) and the eosinophil scatter (MES) between the MDS cases and the control groups.

Out of these six parameters, the significance of lymphocyte volume and neutrophil conductivity is debatable since, these two parameters were found to be significant even between the non MDS cases and controls. This difference however can be explained with the fact that the non MDS category also comprises patients with various illnesses which can considerably alter the morphology of the blood cells.

So only the rest four parameters namely neutrophil volume (MNV) and scatter (MNS), monocyte volume (MMV) and eosinophil scatter (MES) were considered to be of significance.

TABLE 21: Four significantly different CPD parameters in the study.

CPD PARAMETER	**MDS (n-68)**	**NON MDS (n-155)**	**HEALTHY DONORS (n-98)**
Mean neutrophil volume (MNV)	153.76 (Increased)	147.08	148.90
Mean neutrophil scatter (MNS)	125.28 (Decreased)	136.94	132.88
Mean monocyte volume (MMV)	176.43 (Increased)	165.66	164.80
Mean eosinophil scatter (MES)	176.87 (Decreased)	185.50	184.22

The following details can be understood from the table:

1. Both the granulocytes in the study, i.e the neutrophils and the eosinophils showed a significant difference in the scatter values compared to the other groups.

2. The monocyte being an agranulocyte, showed a significant difference only in the volume rather than the conductivity and scatter.

The possible reasons for the findings in this study have been discussed in the preceding sections. In addition to these, the possibility of machine induced changes on the morphology of the blood cells should also be kept in consideration. The automated haematology analyser uses certain reagentssuch asErytholyse and Stabilise to lyse the red blood cells and platelets while performing a differential count on the white blood cells. An isotonic diluent solution is used to dilute the samples for counts and to provide sheath flow to achieve single cell stream for the VCS module to analyse the cells.The Stabilise solution prevents the lysis of the white blood cells and preserves them in the near normal morphology. The changes caused by these solutions may also alter the morphology of these cells by their actions on the plasma membrane and cytoplasm. These changes might be also influenced by the on going cellular changes of apoptosis. Therefore, the CPD parameters may be a reflection of the net effect of the disease associated change added with the reagent induced changes in the analyser.

MICROVESSEL DENSITY IN THE BONE MARROW:

Angiogenesis or the formation of new vesselshas an important roleto play in tumour growth and dissemination.Development of angiogenesis has a prognostic impact in various solid non haematological tumours such as breast carcinoma. Several observers have hypothesized that

the growth potential of primary solid tumours is strictly based on their ability to induce angiogenesis from the surrounding vasculature. The basic reason for this tumour induced neovascularisation is obvious and represents the increased demand of the proliferating tumour cells for more nutrition. Thus an increased vessel density in the tumour bed could be an indirect evidence that reflects the growth potential of the neoplastic cells.

The stimulus for the formation of new vessels is mainly produced by the neoplastic clone of tumour cells themselvesand their interaction with the surrounding stroma.Molecules such as VEGF and bFGF are proangiogenic and are produced by the tumour cells. The most likely stimulus, which triggers the production of these proangiogenic molecules by the tumour cells, is tissue hypoxia. The rapidly proliferating tumour cells are deprived of oxygen in the immediate vicinity.

Long standing hypoxia in the tumour microenvironment upregulates the production of Hypoxia induced factor alpha (HIFa) by the tumour cells.The HIFa molecule is a strong stimulus for the production of pro-angiogenic molecules, which compensate for the tissue hypoxia by alterations in the calibre of blood vessels and formation of new vessels.

Apart from the solid tumours, hematopoietic neoplasms which may be loosely designated as “liquidtumours” are also capable of neovascularisation. One of the early evidence for the presence of enhanced angiogenic activity was documented by Perez-Atayde *et al* in the bone marrow biopsiesof children with ALL.(50) Further studies in cases of multiple myelomas, Acute myeloid leukaemia (AML) and other myeloproliferative disorders (MPD) have also shown a similar feature.

It is widely known that MDS is a preleukaemic condition with a propensity to transform into acute leukaemia. The objective to evaluate the MVD in MDS in this present study was considered based on previous studies done by several investigators who demonstrated an

enhanced degree of angiogenesis in the bone marrow of patients with various haematological malignancies such as multiple myeloma, ALL, AML and CLL(33).

The enumeration of the microvessel density was performed using the antiCD34 immunohistochemical marker which stains the endothelial cells. It is essential to recall that endothelial cells also show positive staining for an array of commonly employed markers such CD31, PECAM-1, VE-Cadherin, Factor VIII etc, which are also known to reliably delineate endothelial cells in tissue sections. Numerous studies done on many cancers have used one or more of these markers for reliably staining the endothelium.

Until now, there is no specific advantage of using a particular marker for the endothelium. Since anti CD34 reliably stains the endothelium with less degree of background staining, this marker was selected for the study. Moreover, in the setting of bone marrow, CD34 is also expressed by blasts. Hence, the number and distribution of these blasts in relation to the endothelial cells can also be possibly noted.

This advantage of using the same marker i.e anti-CD34 to pick up both the endothelial cells and the blasts is not without its own disadvantage. First, in the setting of acute leukaemia or in MDS RAEB-II, the presenceof a high proportion of blasts obscures the endothelial vessels and may create confusion in the number and morphology of the endothelial cells. Second, microvessels, when cut in cross sections simulate blasts. In most instances, one can reliably identify the blasts based upon the granular staining nature, while the endothelial cells show a strong uniform positivity.

In this regard, it is worth mentioning that studies evaluating angiogenesis in breast cancers and other organs have claimed that VE Cadherin and PECAM are unique to endothelial cells and hence are more useful for evaluating angiogenesis. (51)

The present study demonstrated a significant difference in the bone marrow MVD between MDS cases compared to the non MDS control group (p-value:0.000). The mean MVD was higher in the bone marrows of patients with MDS. There was no significant correlation between the MVD and the age, sex or the blast percentage.

Persons who had increased cellularity in the marrow manifested an increase in the mean MVD compared to those with normocellular and hypocellular marrows. The reason for this association is hypothesized as follows: There is an important interdependence between the bone marrow vessels and its vascular bed. Endothelial cells and blood cells develop together and influence each other even from the stages of embryogenesis. The finding of an increased mean number of microvessels in patients with increased cellularity could possibly just represent a normal physiological response to cell proliferation in the setting of a hypercellular marrow.

Studies using PET scan to demonstrate the blood flow in the human bone marrow show that vertebral bodies are the sites of maximum blood flow followed by posterior – superior pelvis and then the other regions of pelvis. The reason for this variation could possibly be due to the presence of actively regenerating cellular marrow at these sites (flat bones)which have an increased physiological demand.(52)

There is evidence that enhanced stimulation of erythropoiesis and granulopoiesis causes increase in blood flow to the marrow. The increased blood flow is characterised more often by vasodilationdue to release of nitric oxide (NO) or by the local accumulation of metabolic end products that have a vasodilatory effect, rather than a true increase in the number of blood vessels per unit area.(52)

In a hypercellular marrow, when the cell turnover is high, an increased production of mature blood cells enter the sinusoids in turn dilating and expanding the sinusoidal network which is

observed as increased number of stainable vessels of varying sizes and an irregular dilated lumen containing blood cells. Thus at this stage, an increased degree of MVD in the cases of MDS with a hypercellular marrow could not be completely ascertained to be a part of the disease.

In addition to the increase in the vessel density, several morphological patterns of vessels were observed in the marrow sections from both MDS and the normal controls. In normal marrow sections, most of the vessels observed were linear and more or less non branching or with occasional branching showing a barely recognizable lumen. In marrow sections from MDS cases, there were non branching linear vessels, branching V or Y shaped vessels, minimally dilated CD34 positive endothelium lined vessels and large irregularly dilated sinusoid like vessels. A point to note was that there was no increase in the normal number of arterioles in the MDS marrows when compared to the control group.

Similar to what was observed in this study, Pruneri *et al* described three basic morphological types of blood vessels in the marrow of MDS patients: large, very irregular vessels with visible lumen, vessels with branched shape and sinusoid like vessels. He also was in agreement with Perez-Atayde *et al* who used the term "endothelial sprouts" for designating small vessels without discernible lumina.(34)(50)

Standard pathological textbooks describe that angiogenesis in tissues can occur as result of outgrowth and sprouting from previously existent vessels or from endothelial progenitor cells of marrow origin. This process of angiogenesis involves vasodilation, increased vascular permeability, proteolytic disruption of the basement membrane and migration of the endothelial cells to the site of the new vessel formation. (30)

It would be logical to consider that these different morphological patterns of blood vessels that were observed represent the different stages of angiogenesis. In most of the marrows

that were assessed, more than one morphological pattern of blood vessel was noted. The morphology also might be reasonably influenced by the stromal cells such as adipocytes and fibroblasts and the degree of reticulin fibrosis.

In several studies that have documented an increase in the MVD in solid tumours, the new vessels that were demonstrated were more or less alike capillaries with a thin, reasonably recognisable basement membrane or atleast a supporting stromal cell adjacent to the vessel. Large irregular dilated sinusoid like vascular spaces were not separately identified. Hence, an increase in the capillary like linear blood vessels can be attributed more or less to the sole purpose of an increased nutrient delivery to the metabolically active tumour cells while the irregular sinusoidal like vessels might represent a normally present sinus which has expanded due to increased haematopoiesis and serves for the exit of blood vessels.

At this juncture, it becomes necessary to highlight another interesting concept proposed by Perez *et al* while explaining the reason for increased angiogenesis in bone marrow biopsies with Acute lymphoid leukaemia (ALL). They propose that in some cases, there may be a system of capillaries in the bone marrow that are not patent in the normal physiological milieu. These capillaries open up in times of increase demand of the hematopoietic tissue by an increased flow of blood, which is controlled by the local bone marrow environment. (50) Thus inthese cases, the increase in the MVD in the marrow simply might result due to more number of functional capillaries rather than formation of new vessels.

In summary, an increase in the number of capillary sized microvessels, with branching and endothelial cells might represent a true neoangiogenesis stimulated by the abnormal bone marrow cells. An increased MVD if interpreted along with other stromal factors such as fibrosis might reasonably predict the behaviour of the hematopoietic tissue that are dependant on them.

The documentation of an increased degree of neovascularisation is not only an important part of histopathological examination, but can also serve to guide patient management and the choices of drugs used for treatment. In malignancies, where angiogenesis has been demonstrated to play a major role and to impact the prognosis, antiangiogenic drugs have been tried as an adjunct to the use of conventional chemotherapeutic drugs.

The most important hematologic malignancy where an increased MVD was demonstrated to affect the prognosis was multiple myeloma. Thalidomide which is a drug used in the treatment for multiple myeloma has antiangiogenic property.(53)

Antiangiogenic drugs target the endothelial cells and reduce the recruitment and proliferation of endothelial progenitor cells. The endothelial cells are generally considered to be genetically very stable compared to the malignant cells which as a rule are genetically very unstable. The malignant cells because of their increased genetic instability have a propensity to rapidly develop new mutations which confers them resistance to conventionally used chemotherapeutic drugs and a growth advantage in comparison with the normal cells.

When the endothelial cells are targeted, it results in rapid reduction in the vessel density which inturn translates into decreased nutrition to the rapidly developing tumour cells. Hence, the tumour cells undergo apoptosis and degenerate due to absence of blood supply. Moreover, the endothelial cells donot acquire rapid mutations to anti angiogeneic drugs due to their genomically stable nature.

CONCLUSION

CONCLUSION:

There are cellular alterations and stromal vascular changes observed in cases of myelodysplastic syndrome (MDS). The cellular changes, which have been documented by observation through light microscopy in the form of dysplasia of the blood cells is reflected in the CPD parameters of the peripheral blood cells in the automated haematology cell counter (Beckman Coulter DXH800). In addition to the differences in the CPD values, there are appreciable differences in the pattern of the scatter plots in persons with myelodysplastic syndrome compared to the non MDS controls.

Out of all the CPD values that were analysed, the mean neutrophil volume (MNV), mean neutrophil scatter (MNS), mean monocyte volume (MMV) and the mean eosinophil scatter (MES) values were found to be significantly different between MDS cases compared to the non MDS cases and the healthy donors. These values can prove to be useful in the identification of MDS when there is peripheral cytopenia(s). Although, at this point, it cannot be concluded that these four above mentioned parameters can be used as a screening or a diagnostic tool for MDS, their usefulness in doubtful cases of MDS with a normal karyotype is not clear and needs further work.

Regarding the stromal vascular alterations, it can be concluded that there is an increase in the mean Micro Vessel Density (MVD) in the bone marrow environment of patients with MDS. The significance of the different morphological patterns that were observed needs further studies and correlation. It is known that the drugs used at present for the treatment of MDS, such as Lenalidomide also exert an antiangiogenic property. The relevance of an enhanced MVD in cases of MDS to that of development of acute leukaemia could not be exactly highlighted by this study.

LIMITATIONS OF THE STUDY:

1. The study did not have a proportionate number of cases in the various subtypes of MDS to assess the significance of the CPD parameters and the MVD among the subtypes.

2. Though a comparison among the various parameters were performed among the low and high grade cases, comparison based on the IPSS scoring would be more clinically relevant.

3. Since the CPD values were obtained from the Beckman DxH 800 automated haematology cell counter, the applicability of these results to that obtained from other cell counters such as Sysmex etc.was not tested.

4. The alterations in the CPD parameters and the MVD after the initiation of the treatment were not assessed.

5. There were 6 cases of MDS in which the blood vessels in the trephine biopsy sections did not stain positively with anti CD34 immunohistochemistry. This could possibly be attributed to improper fixation and tissue processing.

6. A comparison of the MVD of cases with MDS to other haematological conditions such as acute leukaemia, myeloproliferative disorders and aplastic anaemia was not performed due to financial constraints.

APPENDIX:

PROFORMA FOR STUDY:

NAME:

AGE:

SEX:

PLACE:

BIOPSY NO:

HOSPITAL NO:

CLINICAL HISTORY:

Fatigue/ Tiredness/ Bleeding manifestations/ Infections/ Others

(Others - Repeated transfusions/ cigarette smoking)

Family history of blood cancer: Yes / No

LABORATORY TESTS:

RBC Count: (Hb <10 gm/dl)

ABSOLUTE NEUTROPHIL COUNT:(<1.8x10^9/L or > 13x10^9/L)

PLATELET Count: (<100x10^9/L or >450x10^9/L)

UNICYTOPENIA / BICYTOPENIA / PANCYTOPENIA.

BONE MARROW ASPIRATE AND TREPHINE BIOPSY:

CELLULARITY: Hypocellular/ Normocellular/ Hypercellular.

STROMAL ALTERATIONS: Altered topography.

MICROVASCULAR DENSITY: / hpf

RETICULIN FIBROSIS: Grade …….

DYSERYTHROPOIESIS:

Ring sideroblasts.

Nuclear budding / internuclear bridging/ karryorhexis/ multinuclearity/megaloblastoid changes.

DYSGRANULOPOIESIS:

Nuclear hypolobation / Hypersegmentation of nuclei/ Cytoplasmic hypogranularity.

DYSMEGAKARYOPOIESIS:

Micromegakaryocytes/ Hypolobated nuclei/pawn ball appearance.

CATEGORY: ……..

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VELLORE 632 002, INDIA

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Additional Vice Principal (Research)

September 02, 2013

Dr. Vishnu Chandra Kumar. A
PG Registrar
Department of General Pathology
Christian Medical College
Vellore 632 002

Sub: **FLUID Research grant project:**
Characterisation of myelodysplastic syndromes – bone marrow aspirate, trephine biopsy and hemocytometry.
Dr. Vishnu Chandra Kumar. A, General Pathology, Dr. Joy Mammen, Transfusion Medicine and Immunohematology, Dr. Marie Therese Manipadam, General Pathology, Dr. Biju George, Haematology, Dr. Sukesh C Nair, Transfusion Medicine and Immunohematology.

Ref: IRB Min. No. 8315 [OBSERVE] dated 18.06.2013

Dear Dr. Vishnu Chandra Kumar. A,

I enclose the following documents:-

1. Institutional Review Board approval
2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
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CC: Dr. Joy Mammen, Transfusion Medicine and Immunohematology, CMC

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

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